GENETIC EPIDEMIOLOGY AND FAMILIAL RISK FACTORS FOR PARATUBERCULOSIS SEROPOSITIVITY IN BEEF CATTLE

A Dissertation

by

JASON BARRETT OSTERSTOCK

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2007

Major Subject: Biomedical Sciences

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ABSTRACT

Genetic Epidemiology and Familial Risk Factors for Paratuberculosis Seropositivity in Beef Cattle. (December 2007)

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Paratuberculosis is an intestinal infection of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Genetic associations with paratuberculosis have been described in Holstein cattle. The objectives were to describe the association between paratuberculosis status of the dam and her offspring in beef cattle, develop methods to assess familial aggregation of paratuberculosis in cattle of unknown pedigree, and model the paratuberculosis status of beef cattle given familial and herd-level covariates. Texas beef cattle were tested for paratuberculosis using serum antibody and microbiologic culture of feces. Available pedigree data were used to confirm familial relationships.

The association between the paratuberculosis ELISA status of the dam and her offspring was assessed using mixed-effects models. Increased odds of being classified as a “suspect” or greater based on ELISA results were observed if the dam was classified as a “suspect” or greater. A positive linear association was observed between the ELISA value of the dam and her offspring. Analysis of pedigree data using conditional logistic

regression identified ancestors associated with significantly increased odds of being classified as “low positive” or greater.

Microsatellite markers were used to classify cattle into genetically similar groups using allele frequency data. Nine clusters of genetically similar cattle were identified among paratuberculosis test positive cattle, herd matched controls, and pedigreed cattle. Clusters were validated using animals of known pedigree. Increased odds of paratuberculosis seropositivity were observed for some clusters compared to the cluster with the lowest seroprevalence.

A predictive model was developed using a Bayesian framework to assess the association between antibody status of the dam and her offspring adjusted for herd-level risk factors. Predictors associated with highly probable increased odds of seropositivity included herd seroprevalence and herd fecal prevalence of MAP. The association between dam and offspring ELISA status was small (OR 1.35) and not highly probable.

The results of this work support familial aggregation of paratuberculosis ELISA status, but herd-level risk factors appear to be more important in predicting ELISA status. A large proportion of observed serological reactions were not supported by fecal culture results; therefore, observed associations might be limited to humoral responses to *Mycobacterium* spp.

DEDICATION

I would like to dedicate this dissertation to my wife, Dana, and our daughter, Lyvia. They both have been instrumental in helping me complete my graduate program and have undoubtedly endured too many weekends with me still at work and late nights where I was too stressed to be supportive enough of them. Their compassion and unconditional love is more than I deserve and for that I am grateful.

I would also like to dedicate this work to my parents, Earl and Janet Osterstock, who have always been very supportive of my aspiration to become a bovine veterinarian. They always provided me the motivation and support to accomplish my goals. Thank you.

ACKNOWLEDGEMENTS

I would like to acknowledge the hard work and support of my graduate committee; Drs. Geoffrey Fosgate, Allen Roussel, Noah Cohen, and James Derr. In particular, I want to offer special thanks to Drs. Fosgate and Roussel who have invested considerable time and effort in mentoring and direction and have helped me learn how to be a scientist. I would also like to acknowledge the faculty members, house officers, and graduate students in the Departments of Large Animal Clinical Sciences and Veterinary Integrative Biosciences for their support and friendship during my graduate program. Specifically, I want to thank the clinicians in the Food Animal Section and Food Animal Medicine and Surgery Service. They have been instrumental in my development as a food animal clinician, have been very supportive in helping me complete my graduate work, and have helped to develop lifelong friendships.

I would like to recognize all of my clients and, in particular, the herd owners that allowed me to collect samples from their cattle. We spent more than a few days in the heat together and countless hours shuffling records back and forth and it is much appreciated. I also want to thank Dr. Andy Schwartz for his help in processing all of the risk assessments and paperwork for the Texas Voluntary Johne's Disease Program.

There are numerous people who have helped get me through the more technical aspects of this dissertation. I want to acknowledge all of the students that helped with sample collection and data management: Dr. Stephanie Collins, Christopher Merren, Clint Owens, Stephanie Hampe, Kathy Cass, Katie Smyre, Tannetje Crocker, Kristin

Davis, Ashley Berry, Leah Berlinger, Jean Baptiste Auroy, and all the others that spent at least 1 day in the heat chasing Longhorns. I want to thank the staff of the Johne's Testing Center for performing the ELISAs and cultures for this study and specifically Drs. Becky Manning and Mike Collins for their help in Section 2. I want to acknowledge the staff of the DNA Core Laboratory including Amanda Gaither and Claire Kolenda for their patience during my genotyping for Section 2. Special recognition is due to Drs. Chris Seabury and Natalie Halbert for all of the insightful discussion regarding the genotyping project. I also want to recognize Dr. May Boggess for her help in writing code for the genetic distance algorithm used in analyzing the microsatellite data and Dr. Jamie Thompson for helping me get my Bayesian career started.

I need to thank the administrative staff of the Department of Large Animal Clinical Sciences for all of their hard work supporting my research, graduate studies, clinical duties and teaching including Gail Snook, Brandi Tarver, Lora Gonzales, Rachel Sears, and Courtney Marlar. Thanks to my receptionists, Jennifer Pivonka and Jimann Jones, and my technicians, C.W. Haynes and Sarah Knox for putting up with me when I was a bit stressed out on clinics.

Lots of people have contributed in some way to the success of this dissertation. If I failed to mention them specifically, it is my own error and does not reflect the importance of their contribution.

NOMENCLATURE

AFLP	Amplified fragment length polymorphism
AGID	Agar gel immunodiffusion
ANOVA	Analysis of variance
BTA	*Bos taurus* autosome (chromosome)
CARD15	Caspase recruitment domain 15
CF	Complement fixation
CI	Confidence interval
DIC	Deviance information criteria
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
IBD	Identical by descent
IS	Insertion sequence
LRR	Leucine rich repeat
MAP	*Mycobacterium avium* subsp. *paratuberculosis*
MCMC	Markov chain Monte Carlo
MHC	Major histocompatability complex
NOD2	Nucleotide-binding oligomerization domain 2
NRAMP1	Natural resistance associated macrophage protein 1
OD	Optical density
OR	Odds ratio

PCR	Polymerase chain reaction
QTL	Quantitative trait loci
REML	Restricted maximum likelihood
RFLP	Restriction fragment length polymorphism
S:P Ratio	Sample to positive control ratio
SLC11A1	Solute carrier 11A1
SNP	Single nucleotide polymorphism

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1. INTRODUCTION

1.1 Genetic resistance to infectious disease

Infectious diseases of livestock pose a threat to animal health and welfare, human health via the domestic food supply, and economic stability of the agriculture industry as a result of trade restrictions associated with animal disease. Prevention and treatment of infectious diseases attempts to mitigate these threats through the use of antimicrobials, antiparasiticides, and vaccines. In addition to the use of chemotherapeutics, coordinated control programs have been developed for particularly devastating diseases including targeted surveillance for brucellosis in cattle, test and cull programs for bovine tuberculosis, and pasteurization standards for commercial milk. Despite the investment of substantial economic and labor resources in these control programs and a wide array of efficacious chemotherapeutics, infectious disease threats to animal agriculture have not been eliminated. An alternative means of disease control in livestock is selection for animals with natural resistance to infectious disease.

Resistance to infectious disease is largely influenced by several elements of the immune system (Templeton et al., 1988). Following exposure to a pathogen, physical barriers serve as the first opportunity for interaction between the pathogen and host. Receptors on mucosal surfaces may recognize and bind pathogen antigens and initiate immune or pathophysiological responses. Following receptor-mediated attachment, the

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This dissertation follows the style of Preventive Veterinary Medicine.

pathogen has the opportunity to colonize the infected tissue and encounter other cells of the immune system; specifically the innate immune system. Pathogens may be phagocytized by macrophages or neutrophils. Antigens released from these cells may then be presented with 1 of 3 classes of major histocompatability complexes (MHC) which serve to stimulate specific B and T cell responses of the adaptive immune system. Many elements of the immune response are regulated by proteins encoded within the host's genome. This serves as the basis of genetic resistance to infectious disease.

Examples of genetic resistance to infectious disease in livestock have been described for all stages of the immune response and for a variety of pathogens. Innate resistance to colibacillosis has been described in swine lacking the K88 pilus receptor on intestinal enterocytes preventing bacterial attachment (Sellwood et al., 1975; Rapacz and Hasler-Rapacz, 1986). Variability in macrophage function following experimental infection of cattle with *Brucella abortus* has been shown to be associated with resistance to infection (Price et al., 1990). Polymorphisms in the bovine MHC have been associated with resistance to persistent lymphocytosis associated with bovine leukemia virus infection (Lewin and Bernoco, 1986; Xu et al., 1993), dermatophilosis (Maillard et al., 1996), *Boophilus* tick infestation (costa-Rodriguez et al., 2005; Martinez et al., 2006), and vaccine response for Foot-and-Mouth Disease Virus (Garcia-Briones et al., 2000).

Alternatively, one can consider the elements of infectious disease resistance at a more general level and without identification of specific genetic associations or immunological differences. Resistance to infectious disease may be associated with resistance to colonization of the pathogen, persistence of the pathogen, variability in the

development and time to onset of clinical signs, and differences in severity of lesions associated with infection. Each of these elements reflects opportunity for genetic variability and underscores the potential complexity of resistance to infectious disease for most livestock pathogens. It is likely that there are very few infectious diseases where a single gene regulates resistance; instead most are probably controlled by a complex interaction of many genetic and regulatory elements.

Despite the fact that genetic diversity contributes to differences in susceptibility to all infectious diseases, the relative importance of genetic factors compared to environmental or nutritional factors may vary substantially for different diseases and among different species. Selection for livestock with resistant genotypes or targeted surveillance among animals with familial history of infection can only be efficient if genetic differences represent a substantial proportion of disease risk. Therefore, before investing considerable resources in genome-wide association studies or candidate gene research, the genetic influence on susceptibility should be estimated. Measuring the genetic contribution to disease risk is generally accomplished by determining the degree of familial aggregation. Familial aggregation is generally evaluated by comparing the prevalence of disease among family members of affected individuals with the prevalence in the reference population (Liang and Beaty, 2000). This approach is convenient in human populations with generally well defined family structure. Additionally, monozygotic and dizygotic twins and adoptive children can be used to further discriminate between genetic and environmental effects. Livestock populations are

generally more difficult to study because of extensive partial sibship and the aggregation of multiple families within herds.

Traditionally, familial aggregation in livestock has been measured using heritability. Complete descriptions of methods to derive heritability are available. (Khoury et al., 1993; Legates and Warwick, 1990; Thomas, 2004). Practically, heritability represents the proportion of variability in a trait that can be attributed to the effects of genes or interaction among combinations of genes. From a general perspective, heritability is estimated in 2 forms: broad and narrow sense. Heritability in the broad sense is defined as the proportion of variability associated with all genetic factors and narrow sense heritability is defined as the proportion of variability associated with additive genetic effects. Narrow sense heritability more accurately reflects the improvement in traits that can be gained solely through genetic selection because it excludes genetic effects associated with interactions of alleles at the same locus (dominance) and different loci (epistasis). However, estimation of narrow sense heritability requires specific genotypic information or very specific pedigree information for the identification of appropriate relative pairs.

Estimates of heritability can be obtained from a variety of study designs and statistical methods. Interpretation and validity will vary depending on the sample population, model form, and underlying assumptions. The simplest approach is to estimate empirical heritability using simple linear regression with offspring phenotype included as the outcome of interest and parental phenotype modeled as a linear covariate. For the model including the phenotype of a single parent, empirical

heritability is estimated as twice the regression coefficient for the parental phenotype term. Alternatively, an average of the traits of both parents, commonly referred to as the mid-parent value, may be modeled as an independent variable and empirical heritability is equal to the regression coefficient. Empirical heritability provides a very limited evaluation of the influence of genetic factors on a given trait and is limited to quantitative traits.

A preferred approach to estimating heritability is to partition the variance of a given model including parental and offspring phenotypes. This approach is founded in the writings of Fisher (1918) in some of the initial work integrating statistics and genetics. The basic approach is to decompose variability in a trait within a sample population into components attributed to genetic, environmental, and random variability. Models used to identify variance components include analysis of variance (ANOVA) and linear mixed-effects models. An advantage to the use of linear models is that environmental effects may be included in the model to reduce bias in estimates of genetic effects. Additionally, these models may include covariance terms if environmental exposures and parental phenotypes are correlated and interactions to account for genotypic instability across populations in differing environments.

Variance components used to estimate heritability may be calculated from the covariance of phenotypes between varying types of relative pairs. Different relative pairs (ex. parent-offspring, monozygotic twins, dizygotic twins, partial or full sibs, etc.) include varying degrees of shared environmental exposure and differing proportions of alleles shared identical by descent (IBD). Alleles that are shared IBD originate from the

same gamete and the expected proportions are represented by the kinship coefficient (ψ) (ex. 1 for monozygotic twins, ½ for parent-offspring pairs, and ¼ for grandparent-offspring pairs). Methods for determining IBD using genome-wide markers have been described to gain more accurate estimates of IBD and for populations with limited pedigree information (Visscher et al., 2006). Calculation of the covariances may be performed post hoc from the results of ANOVA or may be estimated using restricted maximum likelihood (REML) estimation and mixed effects models. For mixed effects models, the term(s) for which the covariance is estimated (ex. parental phenotype) may be included as a random effect to allow direct estimation of the covariance. To calculate heritability in the broad sense, the variance attributed to genetic effects (σ^2_g) is divided by the sum of σ^2_g, variance due to environmental effects (σ^2_c), and residual variance (σ^2_e). This proportion is multiplied by ψ^{-1} to derive heritability. Narrow sense heritability is calculated by dividing the variance attributed to additive genetic effects (σ^2_a) by the sum of σ^2_a, variance attributed to dominance (σ^2_d), and variance due to epistatic interactions (σ^2_i) and multiplying by ψ^{-1}.

Heritability estimates are often limited and must be interpreted carefully including consideration of study design, the sample population, the linear models used in variance partitioning, and the underlying variability of the trait in the sample population. The influence of the latter can be illustrated easily when considering a purely genetic trait in a population with limited genetic variability or a purely environmental trait in a population with limited environmental variability in the sample population. The heritability estimates for these scenarios should approach 1 and 0, respectively.

However, potential selection bias associated with the limited variability in the sample population would lead to heritability estimates that approach 0 and 1, respectively. The scenario with limited underlying genetic diversity may be of particular importance when considering traits under strong selection resulting in limited genetic variability where heritability is consistently underestimated. Another constant limitation of these approaches is that heritability is derived from measured phenotypes and includes variability in measurement of the phenotype in addition to variability in phenotype. Therefore, heritability based on measured phenotypes will always underestimate the true heritability (Thomas, 2004). Failure to consider correlation of genetic and environmental distributions may lead to overestimation of the true heritability in the population. Inability to detect or account for gene-environment interaction, which can be difficult in some populations, may result in underestimation of the true heritability (Khoury et al., 1993).

Heritability of infectious diseases, measured in the broad sense, generally is less than 25% (Lyons et al., 1991; Welper and Freeman, 1992; Uribe et al., 1995; Zwald et al., 2004; Heringstad et al., 2005; Abdel-Azim et al., 2005) compared to heritabilities growth traits and milk production which have been estimated to be greater than 25% (Seykora and McDaniel, 1983; Welper and Freeman, 1992; Arthur et al., 2001; Koch et al., 2004; Smith et al., 2007). Estimation of heritability in livestock requires knowledge of familial relationships limiting studies to populations with known structure from breed registry pedigrees or detailed production records. Identifying herds with appropriate pedigree information for estimation of heritability may result in selection bias if

management or health factors differ between these herds and those of the entire population to which the estimate may be applied.

1.2 Paratuberculosis in cattle

Paratuberculosis, commonly referred to as Johne's disease, was first described in 1895 in a German dairy cow (Collins and Manning, 2001). The first report of paratuberculosis in the U.S was published in 1908 describing the disease in a Pennsylvania dairy cow (Pearson, 1908). The disease has since been described worldwide. Paratuberculosis has been identified in a host of wild and domesticated ruminants including beef and dairy cattle, bison, goats, sheep, camelids, water buffalo, red and white-tailed deer, and non-ruminant wildlife (Taylor, 1951; Hillermark, 1966; Mohiyuddeeen and Malaki, 1967; Libke and Walton, 1975; Riemann et al., 1979; Power et al., 1993; Belknap et al., 1994; de Lisle and Collins, 1995; Stehman, 1996; Greig et al., 1997; Buergelt and Ginn, 2000; Buergelt et al., 2000; Beard et al., 2001; Mackintosh et al., 2002; Sivakumar et al., 2006). Paratuberculosis is associated with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection. MAP is a slow growing bacterium with significant genetic homology to *Mycobacterium avium* subsp. *avium*, prompting inclusion of MAP as a subspecies of *M. avium* in 1990 (Thorel et al., 1990). The complete genome of MAP has been described (Li et al., 2005). Investigation of genotypic diversity among MAP isolates suggests that there may be some differentiation between isolates obtained from cattle and those obtained from humans and sheep (Motiwala et al., 2003). Further ability to differentiate between cattle isolates may be

achieved based on polymorphisms identified from amplified fragment length polymorphisms (AFLP) within the MAP genome (O'Shea et al., 2004).

Cattle become infected with MAP through ingestion of feces or milk containing MAP (Gilmour et al., 1965; Sweeney et al., 1992a; Streeter et al., 1995; Sweeney, 1996). Additionally, congenital infection has been described due to in utero transmission of MAP (Kopecky et al., 1967; Seitz et al., 1989; Rohde and Shulaw, 1990; van Kooten et al., 2006). In 1 study, 26% fetuses from clinically affected dams diagnosed by use of microbial culture of feces were deemed infected in utero based on microbial culture of fetal tissues (Seitz et al., 1989). A study of congenital infection in subclinically infected cattle found that only 9% of fetuses were infected based on tissue culture (Sweeney et al., 1992b). Embryos harvested from infected cattle are not associated with infection (Bielanski et al., 2006) suggesting that congenital infection may be due to bacteremia during gestation rather than contamination of the oocyte prior to or after ovulation. MAP has also been isolated from the sexual organs and semen of infected bulls and rams, but the significance of this regarding the transmission of MAP is unclear (Larsen and Kopecky 1970; Larsen et al., 1981; Eppleston and Whittington, 2001; Ayele et al., 2004). Cattle are typically infected early in life with an apparent decrease in susceptibility occurring at approximately 1 year of age (Larsen et al., 1975).

Following ingestion of MAP, cattle become infected as the bacterium enters the M cells in the ileum (Momotani et al., 1988). Macrophages located adjacent to the M cells, commonly referred to as dendritic cells, engulf the bacteria. Phagocytosis of MAP by macrophages is mediated by surface receptors including complement receptors

(Schlesinger and Horwitz, 1991; Schlesinger, 1993), mannose receptors (Schlesinger, 1993; Kang and Schlesinger, 1998), and toll-like receptor 2 (TLR-2) (Underhill et al., 1999; Means et al., 1999; Netea et al., 2004). MAP survives in the macrophage by preventing phago-lysosomal fusion and establishes a chronic infection (Coussens, 2001; Tooker et al., 2002). Infected macrophages express interleukin 1 (IL-1), IL-12, and tumor necrosis factor alpha (TNF-α) (Coussens, 2004). These cytokines serve to initiate T cell responses in the Peyers patches including the expression of interferon gamma (IFN γ). Release of IFN γ from T cells activates non-infected tissue macrophages. This activation allows these macrophages to complete phago-lysosomal fusion and destroy the bacterium if subsequently infected. Two major classes of T cells play a central role in the early response to MAP infection and subsequent macrophage activation. Alpha-beta (αβ) T cells expressing the CD3+ surface marker are the predominant T cell subset in adult cattle comprising > 90% of the circulating T cell population (Coussens, 2001). The remaining T cell subset is CD3+ gamma-delta (γδ) T cells. In cattle, γδ T cells predominate in young animals and appear to play a critical role in the initial cell-mediated immune response to MAP infection.

Following macrophage infection with MAP, granulomas begin to form in the intestinal submucosa representing a hallmark pathological lesion associated with paratuberculosis. These granulomas represent an attempt by the immune system to sequester infected macrophages. Release of TNF-α by infected macrophages and T cells stimulates the expression of IL-8, a cytokine associated with initiation of chemotaxis (Coussens, 2001). Additional macrophages and T cells respond to this recruitment,

localizing adjacent to infected macrophages and forming the basis of the granuloma. These lesions, while efficient at localizing the infected macrophage and potentially destroying infected macrophages, are fragile and may intermittently rupture, allowing escape of MAP into the intestinal lumen (Coussens, 2001). These bacteria may be shed in the feces, or may infect additional M cells in the intestinal wall and contribute to the spread of the infection. Similarly, persistently infected macrophages that have migrated to regional lymph nodes may also facilitate granuloma formation.

As the infection progresses, humoral immune responses predominate, but are less effective at controlling the infection and limiting progression of lesions and clinical signs. This transition in immune response is not thoroughly understood. It has been proposed that the chronicity and spread of the infection increases opportunity for development of T helper type-2 (Th2) cells to stimulate antibody formation by circulating B cells. Decreases in γδ T cell populations as the animal matures may also play a role (Coussens, 2001). Further, release of cytokines including IL-10 and transforming growth factor beta (TGF-β) may facilitate reduction of the cell-mediated responses and a transition to predominantly humoral immune responses (Khalifeh and Stabel, 2004). Coincident with this shift in immune response is an expansion of pathological lesions and a gradual onset of clinical signs.

Animals infected with MAP generally go through several stages of infection that have been described on the basis of clinical signs, fecal shedding, and detection of circulating antibody to MAP antigens (Whitlock and Buergelt, 1996). These clinical descriptions have been developed based on observations in dairy cattle and reflect the

progression of typical infections. However, there is undoubtedly variability in the age of onset and duration of the suggested stages of infection among animals and breeds. These differences may reflect underlying genetic variability in onset and efficacy of the immune response in controlling the infection, development of anti-MAP antibodies, or exogenous factors including dose of exposure, nutrition, and stress associated with management and production. Nonetheless, the description of the proposed stages of infection aids in conceptualization of the progression of paratuberculosis. The first stage includes young animals, generally less than 2 years of age, that have persistently infected macrophages and early granuloma formation, but do not have sufficient pathology to induce clinical signs. These animals do not shed MAP in their feces and will not be detected by most routinely used diagnostic tests. The second stage of infection is characterized by more extensive granuloma formation. These animals may intermittently shed MAP in their feces, but similarly do not demonstrate overt signs of infection. Diagnostic test responses in this group may be more variable with early detection of circulating antibodies possible in some animals. The third stage of infection coincides with the development of more extensive pathological changes and the onset of clinical signs. The onset of this stage appears to vary significantly, but appears to develop between 4 and 6 years of age in dairy cattle, although animals as young as 2 years of age may demonstrate clinical signs. Anecdotally, the age distribution for onset of clinical signs in beef cattle appears to be older with clinical signs often not apparent until animals are 6 to 8 years of age. Clinical signs include progressively worsening diarrhea and subsequent weight loss associated with the gradual thickening of the bowel due to

granuloma formation and expansion. Affected animals are afebrile and usually have a normal appetite despite potentially severe diarrhea. Animals in this stage are likely to suffer production losses including decreased milk production and increased incidence of other diseases in dairy cattle (Wilson et al., 1993; Nordlund et al., 1996; Johnson et al., 2001; Chi et al., 2002; Hendrick et al., 2005b; McKenna et al., 2006; Gonda et al., 2007a). Fecal shedding and circulating antibody levels are more consistent and are often readily detectable with a variety of testing methods. The final stage of infection is associated with advanced clinical disease. Affected animals have severe diarrhea and are usually in poor to very poor body condition. Protein loss due to intestinal ulceration and intestinal lymphangiectasia coupled with decreased intestinal absorption associated with enterocyte damage may cause formation of dependent edema secondary to decreased plasma oncotic pressure. Animals in the final stages of infection are generally detectable using available diagnostic tests, but a period of anergy may develop during which circulating antibody levels fall below the analytic sensitivity of conventional tests.

Currently, effective therapies for paratuberculosis infection in ruminants do not exist. Experimentally, monensin sodium has been shown to decrease granuloma formation in mouse models (Brumbaugh et al., 1992). Subsequent studies in naturally occurring infections in cattle demonstrated reduced histopathological lesions in the ileum and liver (Brumbaugh et al., 2000). However, use of monensin sodium as a treatment is prohibited in the U.S. as an extra-label use of a feed additive (AMDUCA, 1994). Recent approval of monensin sodium as a coccidiostat and growth promotant feed additive in dairy cattle has sparked interest in the potential secondary benefit in reducing

fecal shedding in infected cattle. One study has found a significant reduction in fecal shedding of MAP in dairy cattle and a reduction in seropositivity (Hendrick et al., 2006b). A similar study demonstrated a decrease in milk enzyme-linked immunosorbent assay (ELISA) seropositivity following prophylactic administration of monensin sodium to dairy heifers (Hendrick et al., 2006a). Additional mycobacteriocidal drugs that have been investigated include rifampin (St-Jean and Jernigan, 1991; Mondal et al., 1994), isoniazid (Baldwin, 1976; St-Jean and Jernigan, 1991), and clofazimine (Merkal and Larsen, 1973; St-Jean and Jernigan, 1991). Response to these therapies appears to be highly variable depending on the stage of infection when treatment is initiated, and clinical signs generally recur once therapy is has been discontinued (St. Jean and Jernigan, 1991).

Diagnosis of paratuberculosis in cattle can be difficult, particularly during subclinical stages of infection. Clinically affected animals can generally be diagnosed based on characteristic clinical signs and signalment with limited supportive diagnostic testing. Definitive diagnosis in all but the earliest stages of the disease can be made with histopathological examination of biopsy specimens and identification of acid-fast organisms within granulomas of the ileum and associated mesenteric lymph nodes. Methods have been developed for culture of MAP from the feces of infected animals. As stated previously, MAP is a slow growing bacterium and, as such, fecal and tissue culture can be difficult and time consuming. Solid culture media methods that include antibiotic decontamination steps to eliminate non-MAP fecal microbes and mycobactin J supplementation have proven effective, but generally take 16 weeks for proper

interpretation (Matthews et al., 1978; Kim et al., 1989; Whipple et al., 1991). Improved culture methods have been developed that reduce the time necessary for detection of MAP in diagnostic specimens utilizing liquid media and radiometric detection systems (Damato et al., 1987; Collins et al., 1990; Damato and Collins, 1990). Diagnostic tests for MAP DNA have been used independently or in combination with culture methods for the detection of MAP in tissue and fecal samples targeting insertion sequence 900 (IS900) of the MAP genome (Moss et al., 1991; Whittington et al., 1999; Englund et al., 2001). While the rapid turn-around time for most PCR testing of fecal and tissue samples is a distinct advantage over culture methods, PCR is currently the most expensive commercially available test for paratuberculosis and the presence of polymerase inhibitors in the diagnostic sample, specifically in feces, reduces sensitivity by interfering with amplification of MAP DNA.

Tests for circulating anti-MAP antibody are the most commonly used methods for diagnosis, screening, and surveillance of paratuberculosis in cattle. These tests are preferentially used in many testing programs because they are generally cheapest and results are often available in less than 1 week from diagnostic laboratories. Many different methods have been developed for measuring circulating anti-MAP antibody levels including complement fixation (CF), agar gel immunodiffusion (AGID), ELISA, and flow cytometric methods. Additionally, ELISAs have been developed for milk samples and have been shown to possess similar sensitivity and specificity as serum ELISAs at the aggregate-level, but with only moderate agreement between positive test results at the individual animal-level (Hendrick et al., 2005a). A common limitation of

all serological tests for paratuberculosis is poor sensitivity associated with the delay in substantial antibody production due to the prolonged subclinical phase of infection during which humoral immune responses may be limited. Sensitivity estimates of commercially available serological tests vary (Collins et al., 1991; Sockett et al., 1992; Reichel et al., 1999; Nielsen et al., 2001; Dargatz et al., 2001a; Kalis et al., 2002; Collins et al., 2005; McKenna et al., 2005a; McKenna et al., 2005b), but are generally less than 50% when compared to fecal culture as a gold standard. It should be noted that fecal culture also has imperfect sensitivity due to delayed onset of fecal shedding of MAP. A recent comparison of diagnostic testing methods estimated that the sensitivity of serum ELISA was approximately 30% based on methods that do not rely on a gold standard (Scott et al., 2007). Specificity estimates for serological tests for paratuberculosis are generally greater than 95% (Collins et al., 1991; Sockett et al., 1992; Reichel et al., 1999; Nielsen et al., 2001; Dargatz et al., 2001a; Kalis et al., 2002; McKenna et al., 2005a; McKenna et al., 2005b), but studies have shown that in some herds, exposure to non-MAP *Mycobacterium* spp. does reduce specificity of serological tests due to cross-reacting antibodies, specifically associated with M. *intracellulare* and M. *scrofulaceum* (Osterstock et al., 2007; Roussel et al., 2007). Two new serological tests for paratuberculosis have been developed (Eda et al., 2005; Eda et al., 2006; Speer et al., 2006), but have not been subjected to extensive testing in beef or dairy cattle and have not been compared directly to the performance of all commercially available tests. However, preliminary evidence suggests that these tests have improved sensitivity compared to other serological tests. Application of these tests in groups of animals

selected based on known fecal culture or commercially available ELISA status indicated that the sensitivity of these new tests is greater than 95% in animals of varying fecal shedding status (Eda et al., 2005; Eda et al., 2006; Speer et al., 2006). Further, there is evidence that subclinically infected animals may be detected earlier using these tests (Eda et al., 2005). The difference in the apparent sensitivity may be a product of the difference in antigens used. Commercially available ELISAs derive their antigens from MAP cell lysates. These newer tests derive their antigens from the surface of whole MAP bacilli and potentially contain an array of antigens that includes a higher proportion of epitopes that would be primary targets for antibody production following infection. Further investigation of the utility of these tests is necessary including application in field experiments with larger sample numbers. Longitudinal studies comparing the performance of these newly developed serological tests to other commercially available antibody tests, tests for cell-mediated immune responses, and fecal and tissue culture would also be very useful in assessing the accuracy of these assays.

A host of other testing methods have been developed for diagnosis of paratuberculosis, but are not commonly used in herd testing and surveillance programs. Identification of acid-fast organisms in fecal smears and tissue impressions from biopsy specimens may be useful in making a tentative diagnosis. Methods for detection of cell-mediated immune responses to MAP infection have also been used. Delayed-type hypersensitivity reactions can be elicited by intradermal injection of MAP protein extracts and monitoring changes in skin swelling at the injection site, similar to

traditional *M. bovis* testing methods. This method is generally not as specific as other testing methods due to the propensity for false-positive reactions associated with environmental mycobacteria (Kalis et al., 2003). Another assay for cell-mediated immune responses to MAP infection is evaluation of IFNγ production from peripheral mononuclear cells following stimulation with MAP sonicates and a range of positive and negative control stimulants. Studies have shown that IFNγ may be useful in diagnosing paratuberculosis in cattle (Stabel, 1996; Stabel and Whitlock, 2001), but sensitivity does not appear to be consistently better than serological tests and specificity estimates for IFNγ tests have been reported to be lower (Kalis et al., 2003).

Prevalence estimates of paratuberculosis vary widely due to differences in disease classification, diagnostic tests used to establish infection status, sampling methodology, and beef and dairy cattle production systems. Prevalence estimates for dairy cattle range from 2.5 to 17.1% for seropositivity (Braun et al., 1990; Behymer et al., 1991; Collins et al., 1994; Thorne and Hardin, 1997; National Animal Health Monitoring Service, 1997; Adaska and Anderson, 2003; Pence et al., 2003) and were estimated to be 2.9% based on isolation of MAP from lymph nodes of cull dairy cows at slaughter (Merkal et al., 1987). It should be noted that among these prevalence estimates, 2 reported true prevalence based on correcting apparent prevalence for imperfect test sensitivity and specificity (Collins et al., 1994; Adaska and Anderson, 2003), 2 were based on samples collected from cull cattle which might be associated with a higher prevalence than the total dairy cattle population (Merkal et al., 1987; Pence et al., 2003), and 1 is likely subject to a high proportion of false-positive results due to imperfect test

specificity and concurrent exposure to non-MAP *Mycobacterium* spp. in the environment due to geographical location (Braun et al., 1990). Herd-level prevalence from these studies also varies from 22 to 74% (Collins et al., 1994; Thorne and Hardin, 1997; National Animal Health Monitoring Service, 1997). Animal-level prevalence estimates for beef cattle are similarly varied and range from 0.4 to 8.6% (Merkal et al., 1987; Braun et al., 1990; Behymer et al., 1991; Thorne and Hardin, 1997; Dargatz et al., 2001b; Hill et al., 2003; Pence et al., 2003; Roussel et al., 2005), although similar limitations exist for these studies as noted for estimates in dairy cattle. Beef cattle herd-level prevalence estimates range from 7.9 to 43.8% (Thorne and Hardin, 1997; Dargatz et al., 2001b; Roussel et al., 2005); however, the study that estimated a herd-level prevalence of 43.8% was subsequently found to be associated with high exposure to non-MAP *Mycobacterium* spp. and likely includes a substantial number of false-positive test results (Roussel et al., 2007). This is further supported by the fact that only 7.3% of seropositive cattle in this study were positive for MAP based on microbial culture of feces.

The zoonotic potential of MAP is disputed, but MAP has been isolated from tissues and blood of patients with Crohn's disease. Crohn's disease is an inflammatory bowel disease of humans with clinical and histopathological similarities to paratuberculosis in cattle. Patients are generally affected at an early age and the disease is characterized by segmental inflammation of the intestine with the terminal ileum and right colon being most commonly affected. Approximately 50% of Crohn's patients will have intestinal granulomas grossly or histologically as part of the intestinal involvement

(Ellingson et al., 2003). The recent interest in resolving the potential role of MAP in Crohn's disease was stimulated to a large degree by the isolation of *Mycobacterium* spp. from affected tissues. Chiodini et al. (1984) cultured tissue specimens from patients with inflammatory bowel disease and identified 3 unique mycobacterial isolates with similarities to the *Mycobacterium avium – intracellulare* complex and MAP. These isolates were compared to known mycobacteria using restriction fragment length polymorphisms (RFLP) and were found to be most similar to MAP (McFadden et al., 1987). Following these initial reports, many studies have been performed to estimate the association between Crohn's disease and MAP in various populations and using a variety of diagnostic methods. Several studies have reported that higher proportions of Crohn's patients are positive for MAP based on culture (Schwartz et al., 2000; Bull et al., 2003; Naser et al., 2004; Sechi et al., 2005b) or PCR (Schwartz et al., 2000; Sechi et al., 2001; Bull et al., 2003; Naser et al., 2004; Romero et al., 2005; Autschbach et al., 2005; Sechi et al., 2005b) of affected tissues compared to controls, generally including patients with ulcerative colitis, diverticulitis, colon cancer, or non-inflammatory bowel disease. However, several studies have also found no difference in the frequency of identification of MAP by PCR or the identification of acid-fast bacteria in biopsy sections (Fujita et al., 2002; Ellingson et al., 2003; Baksh et al., 2004; Bernstein et al., 2004). A study of U.K. dairy farmers found no association between the diagnosis of paratuberculosis in dairy cattle and the diagnosis of Crohn's in their respective herdsmen (Jones et al., 2006). There are likely many reasons for the inconsistencies in results attempting to study the causal association between MAP and Crohn's. Identification of

acid-fast bacteria in lesions may not be useful in some patients as cell-wall-deficient *Mycobacterium* spp. have been identified in tissues from patients with inflammatory bowel disease (Chiodini et al., 1986). Further, PCR is likely less than 100% sensitive when used on biopsy specimens due to polymerase inhibitors in the intestinal tract or limited numbers of organisms in available tissue sections. It is also likely that differences in study populations and methods of control selection could contribute to inconsistency of results. For instance, Sardinian populations have consistently demonstrated an association between MAP and Crohn's disease (Sechi et al., 2001; Sechi et al., 2004; Sechi et al., 2005a; Sechi et al., 2005b), but this population may represent unique genetic distributions and environmental exposures that would not be applicable to more diverse populations. Further, there is strong evidence of familial aggregation of inflammatory bowel disease in humans. First degree relatives have a 10-fold greater risk of having the same inflammatory bowel disease as their parent for both Crohn's disease and ulcerative colitis (Orholm et al., 1991). Concordance of Crohn's in monozygotic twins has been reported to be between 20% and 44% in contrast to dizygotic twins where concordance has been estimated to be 0% to 6% (Tysk et al., 1988; Reed, III et al., 1992). This suggests that the risk of Crohn's disease is much greater among individuals closely related to an affected patient. Therefore, differences in genetic susceptibility and study population may contribute to the differences in the reported significance of the association between MAP and Crohn's.

Potential roles of MAP include that of a definitive etiologic agent among those individuals with genetic susceptibility for inflammatory bowel disease or an

opportunistic infection secondary to infection by other etiologic agents or auto-immune disease. There is opportunity for exposure to MAP in the human population via the food supply. This may prove to be particularly important if sufficient evidence is collected to warrant classification of MAP as a food safety and human health risk. Retail milk and cheeses appear to be the most likely routes of human exposure to MAP in those without direct contact to infected cattle. Limited evidence suggests that exposure through retail meats is less likely (Jaravata et al., 2007) despite the fact that disseminated infection has been described (Whitlock and Buergelt, 1996). Diagnostic testing of retail milk products in the U.S, U.K, and Czech Republic have identified viable MAP in 3% (Ellingson et al., 2005), 2% (Grant et al., 2002a), and 2% (Ayele et al., 2005) of samples, respectively. A study of retail cheeses in Wisconsin did not isolate viable MAP from cheese products, but did identify MAP DNA by PCR in 5% of samples (Clark, Jr. et al., 2006). The identification and isolation of MAP DNA and viable MAP, respectively, in dairy products reflects potential for MAP to be shed in the milk of infected animals (Taylor et al., 1981; Sweeney et al., 1992a; Streeter et al., 1995) and fecal contamination of raw milk during the milking process. It has also been shown that conventional pasteurization methods can have variable efficacy in eliminating viable MAP from milk depending on pasteurization heat and time parameters (Grant et al., 1996; Grant et al., 1999; Grant et al., 2002b; Stabel and Lambertz, 2004; Stabel et al., 2004; McDonald et al., 2005). Should MAP be definitively implicated as an etiologic agent in Crohn's disease, dairy products would likely be a focus for implementation of food safety measures.

1.3 Genetic risks for paratuberculosis

1.3.1 *Heritability, dam, and genetic effects for paratuberculosis*

Several studies have found evidence to support the existence of variability in genetic susceptibility to paratuberculosis. To date, these studies have been limited largely to dairy cattle, and study designs have restricted to some degree the ability to differentiate the contributions of genetic similarity and common environment to the risk of infection. The most common approach to describing the influence of genetic variation on disease risk has been to estimate the heritability of infection status based on diagnostic test results.

The first contemporary attempt to identify differences in genetic susceptibility was performed using paratuberculosis control program data from Dutch dairy cattle (Koets et al., 2000). The study monitored post-mortem data from cull dairy cows originating from infected dairy farms enrolled in a concurrent paratuberculosis vaccine trial. Cattle were classified as infected based on culture, histopathology, and acid-fast staining of necropsy tissues interpreted in parallel. Heritability of post-mortem status was calculated based on the proportion of the total phenotypic variance attributable to the sire and dam using a probit model which included covariates for vaccination status and herd prevalence at birth. Heritability estimates varied from less than 1% to 9%, with the highest heritability estimates found in a subset of vaccinated animals. The heritability estimate derived by modeling data from vaccinated and unvaccinated animals together

was 6%. This study also estimated the probabilities of infection associated with dam infection status adjusted for prevalence at birth and associated with vaccination status similarly adjusted for herd prevalence at birth. These results support a decreased risk of infection associated with vaccination for paratuberculosis and having an infected dam. The effect of herd prevalence at birth differed in each of these models with, increasing prevalence associated with lower risk in the model containing vaccination status and increasing risk in the model containing dam infection status. These results appear to be counterintuitive given our understanding of the epidemiology of paratuberculosis. We would expect that increasing herd prevalence would be associated with increased risk of infection. We would also expect that animals from infected dams would be more likely to be infected themselves due to vertical transmission across the placenta, ingestion of colostrum or milk from an infected dam, exposure to the dam's feces immediately following calving, and shared genotype associated with susceptibility. The reason for these discrepancies is unclear. One of the likely causes is the effect of vaccination over time on fecal shedding of MAP. Over the 10-year period during which this study was performed, cattle were not vaccinated during years 1 and 2. Further, the known effects of vaccination on fecal shedding would likely decrease environmental contamination with MAP and decrease the risk of ingestion of feces containing MAP. Alternatively, decreases in frequency of culling infected cows due to vaccination may have decreased the opportunity for diagnosis later in the study; particularly since infection status was determined at post-mortem examination and no herd testing program was utilized during the study.

The contributions of dam and sire effects to paratuberculosis test status variability have also been examined in Danish dairy cattle using a milk ELISA (Nielsen et al., 2002b). Using mixed-effects models, the proportion of variability in milk ELISA transformed optical density (OD) attributable to sire identity was 1.9%. In a subset of these animals including only dam-offspring pairs, the proportion of variability associated with sire and dam identity was 6.3% and 7.7%, respectively. A second study in Danish dairy cattle estimated the heritability of paratuberculosis using the same milk ELISA with adjustment for daily milk yield, a factor which has been associated with milk ELISA results (Mortensen et al., 2004). Additionally, this study measured the variability associated with ELISA plate, finding that 21% of the variability in milk ELISA results was associated with ELISA plate. This indicated that laboratory variability may not be negligible in interpreting the results of genetic association studies using milk ELISA results. Heritability in this study was 10% when ignoring plate variability, reported as such to allow direct comparison to previously reported estimates (Nielsen et al., 2002b) that similarly ignored laboratory variation.

Estimation of heritability of paratuberculosis test status in U.S. dairy cattle has been performed in a single published report (Gonda et al., 2006). Gonda et al. (2006) used a sire-daughter design to estimate the proportion of variability in test outcome associated with sire identity. Cows were characterized using radiometric fecal culture and a commercial ELISA interpreted independently and in parallel. Several different mixed-effects models were used to estimate the sire variance used for heritability estimation. In order to increase the number of affected herds in the study, a reduced

ELISA cut-off of S:P ≥ 0.10, corresponding to the "suspect" classification proposed for this ELISA, was used for herd classification (Collins, 2002). The heritability models used the manufacturer recommended cut-off of S:P ≥ 0.25 to classify individual animals as infected. The calculated heritability varied from 9.1% to 18.3% using a Bayesian framework with a prior heritability of 10.2%, the proposed heritability reported by Mortensen et al. (2004). The results of this study support previous estimates of the heritability of paratuberculosis susceptibility based on antibody tests (Mortensen et al., 2004). The influence of the prior probability for heritability used in this study may have contributed to the similarity in the results as it is not clear if a non-informative prior was also evaluated. It is likely that there was some selection bias in the samples used for the estimation of the heritability in the U.S. cattle population. Herds were selected based on serological status and this study utilized a convenience sample of herds enrolled in another study of the genetic susceptibility of cattle to MAP infection. This selection procedure resulted in a group of daughters from a limited number of sires with very little relatedness among sires. A difference between estimation of heritability in this study compared to other estimates for heritability of paratuberculosis and other traits is the use of sire identity rather than sire ELISA status in the model. However, this would not be expected to substantially alter the heritability estimate in a model that included the parent as a random effect.

An alternative approach to estimating the disease risk associated with infection status of the dam is to estimate the odds of test positivity associated with test status of the dam or sire. In a study of California dairy cattle, Aly and Thurmond (2005) estimated

that the odds of seropositivity in Holstein cattle were 6.5 times greater for animals with a seropositive dam. Further, they estimated that 84.8% of the risk of seropositivity was attributable to having a seropositive dam. There are, however, several aspects of this study that may affect extrapolation to other herds or breeds. First, the results of the ELISA were interpreted as positive if the S:P ratio was ≥ 0.35. The results using the manufacturer's recommended cut-off of 0.25 suggested that the odds of seropositivity and attributable fractions were less than those reported for the higher cut-off. Second, the animals in the study were born over a period of several years during which management changes occurred including improved calving hygiene, feeding of pasteurized colostrum, and reduction of exposure of the calf pens to flush water from the adult cows and milking parlor. The odds ratios reported for the association of dam and daughter serological status are adjusted for exposure to the flush water, but it is possible that the additional changes in management and calf health may have resulted in bias. Finally, the results of this study were all from 1 farm and may not be indicative of the true association in all similarly managed dairy farms.

To date, there has been limited effort to characterize potential associations between the paratuberculosis status of the dam and her offspring dam and genetic effects in beef cattle. One report found that *Bos indicus* cattle had a higher prevalence of seropositivity compared to other purebred beef cattle breeds in Texas (Roussel et al., 2005). The odds of seropositivity in this study were 17 times greater for *Bos indicus* cattle compared to *Bos taurus* breeds, adjusted for environmental risk factors, herd management, and history of animals with characteristic clinical signs on the ranch. It is

unclear if this result is associated with increased susceptibility to infection, increased propensity to develop antibodies in response to MAP infection, or due to geographical differences in exposure to non-MAP *Mycobacterium* spp. that appears to be correlated with the distribution of *Bos indicus* cattle breeders in Texas. A study of paratuberculosis seropositivity in a multibreed herd of beef cattle similarly identified increased antibody levels in Brahman cattle and cattle with increasing proportion of Brahman lineage (Elzo et al., 2006). In this study, IDEXX ELISA S:P ratios were converted to linear scores corresponding to proposed classifications for this ELISA (Collins, 2002). Mixed-effects modeling identified a positive linear effect of increasing Brahman fraction of the cow and predicted ELISA score. However, the potential for false-positive serological reactions in these cattle was not addressed. Further, the use of ELISA scores rather than the observed S:P ratios may have resulted in different observed effects than the true association between antibody level and breed, although the nature of this potential effect is uncertain.

Identification of disease-associated genotypes in ruminants remains in its infancy in paratuberculosis research. A study of a limited number of sheep identified polymorphisms associated with candidate genes, specifically solute carrier 11a1 (SLC11A1; formerly natural resistance associated macrophage protein 1 (NRAMP1)) and MHC loci, and paratuberculosis status (Reddacliff et al., 2005). More recently, a genome-wide scan in dairy cattle has identified a quantitative trait locus (QTL) associated with paratuberculosis status on *Bos taurus* autosome 20 (BTA20) (Gonda et al., 2007b). This QTL was identified after scanning the bovine genome using

microsatellites in several Holstein sire families. Chromosomal regions significantly associated with paratuberculosis status based on pooled sample genotyping were subjected to interval mapping on a subset of samples. The results of interval mapping were evaluated against the probability of infection based on post-test odds of paratuberculosis infection using likelihood ratios for different ELISA values estimated for the IDEXX ELISA (Collins, 2002). It should be noted that this QTL was only identified in 1 Holstein sire family and the location was not precisely estimated as evidenced by the width of the reported confidence intervals for the mapped location. Microsatellites in linkage with reported candidate genes have also been evaluated for association with paratuberculosis status in German dairy cattle (Hinger et al., 2007). Comparison animals were half-sibs matched by age and herd. No association between microsatellite genotype and paratuberculosis test status was observed. However, rare alleles were aggregated into a single group within each microsatellite which may have obscured associations of some rare alleles with paratuberculosis ELISA status. Additionally, the authors did not adjust for multiple comparisons, although this would not be expected to alter the conclusions regarding statistical significance in the present study.

A whole genome single nucleotide polymorphism (SNP) association study was performed in the same group of dairy cattle used in the microsatellite study by Gonda et al. (2007b) using a commercially available SNP array (Gonda, 2006). Numerous SNPs were identified across the genome that were associated with paratuberculosis status, as would be expected with over 2,000 SNPs genotyped. Interestingly, BTA 20 contained 1

of the largest proportions of significantly associated SNPs of any chromosome; this chromosome also contained the aforementioned QTL. There are several limitations to this genome-wide SNP study. First, the samples were performed on pooled DNA samples and not individual animals. There has been limited work utilizing pooled samples in SNP arrays and there may be differences in genotyping error rates between the 2 approaches. Additionally, the SNP genotypes are not independent and uncorrected P values for comparison of allele frequencies between infected and uninfected pools would be inappropriate. In this study, Gonda et al. utilized Bonferroni adjustment for multiple comparisons to establish an adjusted P value for determination of significant associations. This approach generally would be considered overly conservative and would likely be so restrictive as to falsely classify some associations as not significant. Attempts to use false discovery rate were attempted, but resulted in an unmanageable number of significant associations. It is possible that alternative methods of calculation of false discovery rate or other methods for analysis of correlated data may have been more appropriate.

1.3.2 *SLC11A1 and paratuberculosis resistance*

Genetic resistance to intracellular pathogens including *Mycobacterium bovis*, *Salmonella typhimurium*, and *Leishmania donovani* has been identified in mice (Plant and Glynn, 1976; Bradley, 1977; Gros et al., 1981). The gene associated with apparent phenotypic resistance to infection with these pathogens was originally referred to as Bcg/Ity/Lsh corresponding to these 3 pathogens, but has since been identified as

NRAMP1 (Vidal et al., 1993), also referred to as solute carrier 11a1 (SLC11A1). This gene, located on autosome 2 in cattle and humans and 1 in mice, is expressed in macrophages and tissues of the reticuloendothelial system. The polymorphism associated with resistance to infection with intracellular pathogens in SLC11A1 was localized to an amino acid substitution at position 169 in the second transmembrane protein domain with glycine at this position being associated with resistance and aspartic acid associated with susceptibility (Vidal et al., 1993; Vidal et al., 1996). Subsequently, additional polymorphisms have been identified in the SLC11A1 gene (Horin et al., 1999; Coussens et al., 2004), including a variable number tandem repeat in the 3' untranslated region (3'UTR) (Horin et al., 1999) that has been associated with resistance to *Brucella abortus* infection (Adams and Templeton, 1998; Barthel et al., 2001), another intracellular bacterial pathogen. Recently, a polymorphism in the equine SLC11A1 gene has been associated with resistance to *Rhodococcus equi* infection in foals, an intracellular pathogen with similarities to MAP including infection limited to young animals and inhibition of phagolysosomal fusion in infected macrophages (Halbert et al., 2006). The mechanism of resistance to infection with intracellular pathogens associated with SLC11A1 polymorphism has not been fully elucidated. The second transmembrane domain plays a role in iron transport (Sechi et al., 2006) and SLC11A1 expression in activated macrophages is associated with the increased production of oxygen and nitrogen radicals that are responsible for killing intracellular pathogens during phagolysosomal fusion (Vidal et al., 1993).

Studies evaluating SLC11A1 polymorphism and mycobacterial diseases have identified inconsistent associations between this gene and apparent resistance to infection. Studies of *M. tuberculosis* infection in humans and SLC11A1 genotype have demonstrated increased odds of infection among patients with 3'UTR polymorphism (Bellamy et al., 1998), associations between SLC11A1 polymorphisms and pediatric tuberculosis (Malik et al., 2005), and associations with disease severity (Zhang et al., 2005). However, SLC11A1 was not associated with *M. avium* complex pulmonary infections (Koh et al., 2005) and susceptibility alleles were dominant (Bellamy et al., 1998), in contrast to observations in mice where resistant alleles were dominant (Vidal et al., 1993). It should be noted that these studies were performed in ethnically diverse populations and the observed differences in susceptibility and mode of inheritance may reflect differences associated with under-represented alleles. A study in cattle found no association between polymorphism in the 3'UTR and *M. bovis* infection status (Barthel et al., 2000).

The potential role of SLC11A1 in paratuberculosis resistance has been examined in experimentally infected mice and Crohn's patients with positive PCR results for IS900. In mice, SLC11A1 resistant phenotype and MAP resistant phenotype were in exact agreement, suggesting that resistance to paratuberculosis in mice was either associated with the SLC11A1 gene or genes at closely linked loci (Frelier et al., 1990). Similarly, the C57BL/6 and C3H/HeN strains of mice, SLC11A1 susceptible and resistant, respectively, were shown to display histological and microbiological differences in resistance to experimental MAP infection (Veazey et al., 1995). Chiodini

and Buergelt (1993) also observed differences in persistence of MAP in infected tissues and quantitative bacterial isolation over time from infected organs with different strains of mice, although the SLC11A1 resistance status of those mice was not reported. Three studies have examined the association between SLC11A1 and Crohn's disease (Hofmeister et al., 1997; Stokkers et al., 1999; Sechi et al., 2006). One study identified an association between SLC11A1 and Crohn's disease in a comparison between patients with Crohn's, ulcerative colitis, and controls using 2 microsatellites linked to the SLC11A1 gene (Hofmeister et al., 1997). However, this study did not have sufficient power to compare all haplotypes for these 2 loci and Crohn's status because parental genotype was unknown. They simply showed that allele frequency differed among the patient and control populations. A second study did not identify an association between SLC11A1 polymorphism and Crohn's status in a population of patients in The Netherlands. It should be noted that this study also evaluated another potential candidate gene for Crohn's susceptibility and found that the alleles were fixed in this population, suggesting that limited genotypic diversity in the population may have affected results. In contrast to these 2 studies, significant associations were identified for a base substitution in the SLC11A1 gene and Crohn's disease, adjusted for identification of the MAP associated IS900 sequence in affected tissues (Sechi et al., 2006). The adjusted odds of Crohn's disease in this Sardinian population were 50.8 times greater in patients with a C/T substitution at position 823 and 5.6 times greater for patients with a deletion downstream of exon 15, adjusted for the presence of IS900 in affected tissues and the presence of the other polymorphisms using multivariable analysis methods. This model

was selected based on likelihood ratio tests of full and reduced models. Significant associations were not observed between polymorphisms at either loci and identification of MAP by PCR, but these data and changes in coefficients in the multivariable logistic models after removing MAP PCR status from the model were not reported. This would suggest that MAP and genetic effects are independent; however, the potential for limited genetic diversity and perhaps unique environmental exposures in this population should again be noted.

The effect of SLC11A1 genotype on paratuberculosis infection risk in cattle remains unknown. Clearly, varied results have been obtained in studies evaluating SLC11A1 genotype and mycobacterial infections in cattle and other species. It is likely that SLC11A1 plays a role in risk of infection in some animals and may be associated with severity of infection and onset of clinical signs. However, if the genotypes associated with resistance to brucellosis are also associated with paratuberculosis resistance, the findings of Paixão et al. (2006) would be contrary to our current understanding of MAP infection. For instance, their study found that 100% of Holsteins and 31.2% of Zebu cattle possessed the brucellosis resistant genotype. The prevalence of paratuberculosis in Holsteins worldwide would not support the role of SLC11A1 in paratuberculosis resistance if the resistant genotype is the same for brucellosis and paratuberculosis and the animals sampled in this study were representative of allele frequencies of the Holstein breed in general. However, 2 reports have suggested increased susceptibility to paratuberculosis seropositivity in *Bos indicus* cattle compared to other beef breeds (Roussel et al., 2005; Elzo et al., 2006), potentially supporting the

role of SLC11A1 if 68% of *Bos indicus* cattle do in fact carry susceptibility alleles as described by Paixão et al.

1.3.3 *NOD2/CARD15 and paratuberculosis resistance*

Another gene that has been studied for associations with resistance to inflammatory bowel disease in humans and MAP is caspase recruitment domain 15 (CARD15). First identified as inflammatory bowel disease locus 1 (Cavanaugh, 2001), it was later referred to as nucleotide-binding oligomerization domain 2 (NOD2) and more recently CARD15 (Lesage et al., 2002). CARD15, much like SLC11A1, is expressed in monocytes and has been shown to play a role in nuclear factor κB (NF-κB) stimulation (Ogura et al., 2001). Deletion mutations in the leucine rich repeat (LRR) region of the CARD15 gene are associated with inappropriate NF-κB activation and subsequent granuloma formation (Ogura et al., 2001). Additionally, 1 frameshift mutation and 2 base substitutions have also been identified and investigated for association with inflammatory bowel diseases; however, 1 of the base substitutions is in linkage disequilibrium with the other 3 mutations (Lesage et al., 2002). Odds of Crohn's disease in patients with familial history of inflammatory bowel disease were 2.6, 2.7, and 6.3 times greater in patients with the frameshift mutation and 2 base substitutions, respectively (Cuthbert et al., 2002). Similar associations were noted in another study of inflammatory bowel disease patients (Lesage et al., 2002). Additionally, patients homozygous for any of these mutations were at increased risk of stenosis in affected bowel and were associated with decreased odds of lesions in the transverse colon, left

colon, and rectum (Lesage et al., 2002). A study of CARD15 polymorphism found that the odds of Crohn's in patients with at least 1 of the 3 mutant alleles were 4.1 times greater than patients with no mutations present (Sechi et al., 2005a). This study also identified 8 times greater odds of MAP infection in Crohn's patients compared to controls and 7 times greater odds of MAP infection among those Crohn's patients with at least 1 mutant allele. It should be noted that these odds ratios are not those that were reported, but were calculated based on data presented in tabular form and statistical discrepancies pointed out in response to this study (Sieswerda and Bannatyne, 2006).

The association between CARD15 and paratuberculosis in cattle has also been studied (Taylor et al., 2006). Taylor et al. described sequence variability in CARD15 among several different breeds and compared haplotypes and individual SNPs with paratuberculosis status in cattle. This study localized CARD15 to bovine autosome 18 and identified 23 SNPs with 21 haplotypes among the breeds evaluated. They also showed that cattle and humans shared 81.2% amino acid sequence homology. No associations were found between CARD15 haplotypes and paratuberculosis status. However, 1 SNP allele was fixed in all cases and was present in only 50% of the comparison group, suggesting that either this SNP was associated with a recessive trait for susceptibility or MAP exposure was not uniform in all comparison animals if the allele was dominant.

1.4 Summary and conclusions

Despite several decades of research and control programs, paratuberculosis remains a problematic disease for the cattle industry. Limitations of currently available diagnostic tests have hindered efforts to eradicate the disease and have hampered research efforts. It is unlikely, given the progression of immunological response and clinical disease, that more reliable diagnostic methods will become available in the near future. Control programs in the beef and dairy cattle industries, motivated by economic losses and the potential zoonotic role of MAP, must seek alternative means beyond test-based culling. The development of improved vaccines for paratuberculosis may prove to be most beneficial in reducing fecal shedding of MAP and subsequent infection in susceptible animals. However, vaccination using commercially available products is unlikely to be applied on a broad scale in the U.S. due to the potential interference of commercially available vaccines with tuberculosis testing (Kohler et al., 2001; Muskens et al., 2002). In lieu of improved diagnostic testing methods and the development of commercially available vaccines suitable for use in the presence of existing tuberculosis testing programs, alternative means of control must be utilized, presently based predominantly on reduction of environmental contamination and improvements in herd biosecurity (USDA, 2002).

The investigation of genetic risk factors for paratuberculosis has the potential to identify additional opportunities for control of paratuberculosis. Markers associated with disease loci could be used in genetic selection programs. Identification of genes associated with paratuberculosis status may also contribute to the understanding of the

pathophysiology of the disease. This knowledge may lead to the identification of alternative means of diagnosis and control, elucidation of the complex progression of immunological response to infection with MAP, and identify opportunities for improved treatment or paratuberculosis and similar inflammatory bowel diseases in comparative species. Further, the estimation of the risk of paratuberculosis infection in offspring of infected animals can help to make culling decisions prior to diagnostic testing or development of fecal shedding in light of the proposed risk and economic value of the animal. Presently, there is limited information available regarding genetic risk factors for paratuberculosis and associations between infected parents and offspring, particularly in beef cattle. The development of advanced technologies for identification of genetic associations with infectious disease will likely fuel this area of research. However, the potential effect of genetic selection for disease resistance on paratuberculosis prevalence within infected herds or globally remains to be determined. Given the estimates of heritability of infection status to date, it is unlikely that genetic selection alone would substantially reduce prevalence in the short-term. The benefit of genetic selection would likely be most effective at reducing the proportion of the population susceptible to infection in concert with improved diagnostic methods and control programs such that the disease could be more efficiently controlled in the long-term. To better assess the benefit of genetic selection for paratuberculosis resistance in beef cattle, the degree of familial aggregation of paratuberculosis in beef cattle must be assessed. The degree of familial aggregation of paratuberculosis in beef cattle will also serve as the foundation to support future studies for disease susceptibility loci.

The objectives of this dissertation are to characterize the degree of familial aggregation of paratuberculosis in beef cattle and to assess the impact of genetic factors on disease risk. This information could help efforts to control paratuberculosis in beef cattle herds in several ways. First, it will provide producers and veterinarians with a measure of the risk of infection in offspring associated with having an infected dam or sire. Second, it will provide an estimate of the heritability of infection in beef cattle. These measures of familial aggregation will serve to support future efforts to identify disease-associated loci. This dissertation will also develop new methods for assessing familial aggregation in beef cattle populations in the absence of pedigree information. This information will provide a platform for future investigations of familial aggregation of additional infectious diseases of importance to the beef cattle industry. The goal of this work will be to expand methodologies for studying genetic epidemiology in beef cattle populations and develop risk measures for paratuberculosis in beef cattle that can aid producers and veterinarians in controlling paratuberculosis.

2. FAMILIAL ASSOCIATIONS WITH PARATUBERCULOSIS ELISA RESULTS IN TEXAS LONGHORN CATTLE

2.1 Introduction

Paratuberculosis, commonly referred to as Johne's disease, is a chronic bacterial infection of ruminants caused by infection with *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Paratuberculosis control programs often utilize serological tests for rapid and cost-effective screening of animals (Collins et al., 1991; Sweeney et al., 1995; Dargatz et al., 2001a; Collins et al., 2005; McKenna et al., 2005a). These tests measure circulating antibody directed at antigens presumed to be associated with paratuberculosis. Prevalence estimates for MAP antibody in beef cattle range from 0.4% to 8.6% (Thorne and Hardin, 1997; Dargatz et al., 2001b; Hill et al., 2003; Pence et al., 2003; Roussel et al., 2005). A study of beef cattle in Texas estimated that 3% of animals were seropositive for MAP and 7.9% of herds contained at least 1 seropositive animal (Roussel et al., 2005). Subsequent studies have found that enzyme-linked immunosorbent assays (ELISA) for paratuberculosis are less specific in some herds than has been previously reported due to exposure to non-MAP *Mycobacterium* spp. (Osterstock et al., 2007; Roussel et al., 2007). Herds with similar exposure must use confirmatory tests including microbial culture of feces and polymerase chain reaction (PCR) assay for insertion sequence IS900 in bovine feces to identify infected animals.

Recent reports in dairy cattle have suggested that there is a familial predisposition to MAP infection (Koets et al., 2000; Nielsen et al., 2002b; Mortensen et

al., 2004; Aly and Thurmond, 2005; Gonda et al., 2006). Given the complex nature of this disease, it is likely that genetic susceptibility to infection is multifactorial with differing genotypes associated with establishment of infection, progression of clinical signs, and immunological response to MAP. Studies have estimated the heritability of paratuberculosis serological status in dairy cattle to be between 9% and 15.3% (Mortensen et al., 2004; Gonda et al., 2006) and the heritability of MAP infection, diagnosed via post-mortem tissue culture and histopathological examination, to be 6% (Koets et al., 2000). Studies have also reported potential associations with polymorphisms at candidate gene loci and paratuberculosis status in sheep (Reddacliff et al., 2005), mice (Veazey et al., 1995), and cattle (Gonda et al., 2007b), although putative disease loci have not been established. Two reports have identified breed predisposition for paratuberculosis ELISA results with increased odds of seropositivity in *Bos indicus* cattle breeds (Roussel et al., 2005; Elzo et al., 2006).

Anecdotally, many producers have assumed that the offspring of dams with paratuberculosis are at increased risk of infection compared to offspring of uninfected animals. Potential contributions to this risk include transmission of MAP from the dam to her offspring in utero (Seitz et al., 1989; Sweeney et al., 1992b) or through ingestion of contaminated milk (Sweeney et al., 1992a; Streeter et al., 1995), increased risk of exposure to feces containing MAP from the dam, co-residence in a contaminated herd environment, and shared genotypes associated with susceptibility to infection. Because of this perceived risk, a common practice in some herds is for the offspring of animals with positive paratuberculosis ELISA or fecal culture to be culled prior to test age or

irrespective of offspring test status. This culling practice may cause substantial economic losses, particularly in purebred cattle operations, where the potential loss of genetic value from unnecessary culling of an uninfected animal may be considerable. Further, in herds with exposure to *Mycobacterium* spp. in the environment that may cause false-positive results on paratuberculosis ELISAs, this practice may compound the economic losses associated with misdiagnosis. The objectives of this study of Texas Longhorn cattle were to examine the pedigrees of animals to identify associations between ancestors and paratuberculosis ELISA status, describe the association between paratuberculosis ELISA results of the dam and her offspring, and estimate the proportion of variability in paratuberculosis ELISA results attributable to the dam in a population of cattle with potential for exposure to non-MAP *Mycobacterium* spp.

2.2 Materials and methods

2.2.1 *Animals*

Texas Longhorn cattle breeders within 150 miles of Texas A&M University in College Station, TX were identified from a breed directory and were surveyed by mail. The survey questionnaire collected information regarding herd size, source of replacements, history of paratuberculosis test-positive animals or animals with undiagnosed chronic diarrhea, willingness to participate in the Texas Voluntary Johne's Control Program, and willingness to participate in this research project (Appendix A). From the herds that were willing to participate in both the state control program and the

research project, all herds reporting a history of test-positive cattle or cattle with clinical signs compatible with a diagnosis of paratuberculosis and all herds with ≥ 15 test eligible animals were selected for sampling. Within herds selected for sampling, all animals ≥ 2 years of age were selected for diagnostic testing. The sample size was estimated assuming a seroprevalence of 1% among cattle from seronegative dams and the desire to detect a minimum odds ratio of 5 with 95% confidence and 80% power. These calculations resulted in a necessary sample size of 710 cattle. All herd owners enrolled in the study provided written consent approved by the Clinical Research Review Committee of the Texas A&M University College of Veterinary Medicine and Biomedical Sciences.

2.2.2 *Diagnostic testing*

Blood and fecal samples were collected from the coccygeal vein and rectum, respectively, from all animals selected for sampling. Fecal samples were submitted and processed for radiometric fecal culture in liquid medium as previously described (Collins et al., 1990). Briefly, the medium was supplemented with mycobactin J, egg yolk suspension, and antimicrobials. Fecal samples were decontaminated with 1.0% hexadecylpyridinium chloride and concentrated via filtration. The resulting filter membrane was placed into radiometric culture medium (BACTEC 12B, BD Diagnostic Systems, Franklin Lakes, NJ) and evaluated weekly for growth using an ionization detector (BACTEC 460, Johnston Laboratories, Townson, MD). A PCR assay for the IS900 gene insertion element was used to identify MAP when acid-fast organisms were

isolated. Mycobacterial isolates negative for IS900 were classified as non-MAP mycobacteria and further characterization was not performed. Serum was separated from each blood sample and submitted for evaluation using 2 commercially available paratuberculosis ELISA kits: ELISA-A (Herdchek®, IDEXX Laboratories Inc, Westbrook, ME) and ELISA-B (Parachek®, Prionics, Schlieren, Switzerland). Test results for ELISA-A were converted to S:P ratios by taking the difference between the sample optical density (OD) and the mean of duplicate negative control ODs and dividing by the difference between the means of the positive and negative control ODs. Individual sample results were dichotomized into positive or negative using the S:P ratio cut-off of 0.25, as recommended by the manufacturer, and using the classification scheme proposed by Collins et al. (Collins, 2002). Results from ELISA-B were classified as positive if the difference between the assay cut-off value and the sample OD multiplied by 100 was greater than 0. The cut-off value for this assay is determined by adding 0.1 to the mean OD of the duplicate negative controls on each run of the assay.

2.2.3 *Analysis of pedigree data*

Pedigrees were collected for all registered animals and were used to identify familial relationships among the sampled animals. Up to 3 generations of familial data were recorded using the registration number to reference the individual and query relationships among animals. From the list of all ancestors identified in the pedigrees, those ancestors that appeared ≥ 20 times among all pedigrees were identified and coded

as categorical exposure variables. Ancestors were coded alphabetically from A to Y in descending order of frequency among all pedigrees.

Herd of residence had the potential to influence the data analysis in 2 ways; as a confounder of the association between ancestors and offspring paratuberculosis test status and as a clustering variable violating the assumption of independence yielding inappropriate standard errors. Preference was given to controlling confounding if present to provide unbiased point estimates. Mixed-effects logistic regression was used to estimate the association between the ELISA status of the dam and the ELISA status of her offspring. Results were compared to estimates of effect derived from conditional logistic regression matched by herd and ordinary logistic regression without a herd effect to identify potential confounding. If confounding was insufficiently controlled using mixed-effects models, the results of the conditional logistic regression model were reported.

The association between presence of a given ancestor in the pedigree and offspring paratuberculosis ELISA status was estimated. The association between the number of times a given ancestor appeared in a pedigree due to inbreeding and offspring paratuberculosis ELISA status was also estimated. The number of times an ancestor appeared in the pedigree was modeled as a categorical variable and absence of the ancestor served as the referent group. For these analyses, all ancestors with $P < 0.25$ in the univariate analysis were selected for multivariable analysis to adjust for the presence of other ancestors. These analyses were performed independently for each of the paratuberculosis ELISA kits. All statistical analyses were performed using commercially

available software (Intercooled Stata version 9.2 for Windows, StataCorp LP, College Station, TX) and $P < 0.05$ was used to identify statistical significance.

2.2.4 *Dam effects*

Dam-offspring pairs were identified based on the pedigree analysis where the paratuberculosis ELISA status was known for both the dam and her offspring. Herd was evaluated similarly for control of confounding of the association between the dam and offspring paratuberculosis ELISA status using mixed-effects, conditional, and ordinary logistic regression. The odds of having an increased S:P ratio if the dam had a similarly increased S:P ratio relative to the odds associated with dams with lower S:P ratios was calculated using several S:P ratio cutoffs, adjusting for age of offspring. Serum ELISA S:P ratio cut-offs used to dichotomize test results corresponded to the breakpoints associated with a previously reported classification system for this ELISA. (Collins, 2002).

The linear association between dam and offspring natural logarithm (log) transformed S:P ratios for ELISA-A was examined using generalized linear latent and mixed models (GLLAMM) (Rabe-Hasketh et al., 2005) to account for correlation of observations within herd and adjusted for age of offspring. Herd was evaluated as a potential confounder of the linear association between dam and offspring transformed S:P ratio. The proportion of variability in transformed offspring S:P ratio associated with the transformed S:P ratio of the dam was modeled using linear mixed-effects models as an indication of the degree of heritability of paratuberculosis ELISA status. For this

analysis, age was modeled as a fixed effect and both herd of residence and dam transformed S:P ratio were modeled as random effects. Inclusion of both herd of residence and transformed dam S:P ratio as random effects allowed partitioning of the total variance into that contributed by the dam, herd, and residual error. The proportion of variability attributable to the dam was calculated as the variance associated with the random effect term for transformed dam S:P ratio divided by the sum of the variances associated with the transformed dam S:P ratio, the random effect for herd, and the residual variance. Heritability was estimated by multiplying this value by the inverse of the proportion of alleles shared identical by descent between the 2 individuals (Khoury et al., 1993); in this case 2 since a dam and her offspring would share ½ of their alleles identical by descent. All statistical analyses were performed using commercially available software (Intercooled Stata version 9.2 for Windows, StataCorp, College Station, TX) and statistical significance for point estimates was determined at the $P < 0.05$ level.

2.3 Results

Surveys were mailed to 762 Texas Longhorn breeders in central and coastal Texas. The total number of respondents was 147 (19.3%) from which 17 herds containing a total of 720 animals ≥ 2 years of age were selected for sampling. Of the 720 animals sampled, 715 had complete diagnostic test results (Table 1) including both ELISAs and fecal culture. Among these 715 animals, 4 (0.6%) were fecal culture positive for MAP, 77 (10.7%) had other *Mycobacterium* spp. cultured from their feces,

36 (5.0%) were positive on ELISA-A, and 24 (3.4%) were positive on ELISA-B. Of those animals with negative fecal cultures, 24 (3.8%) were seropositive with ELISA-A and 12 (1.9%) were seropositive with ELISA-B. Within-herd prevalence ranged from 0.0 to 2.9% for MAP on fecal culture, 0.0 to 42.6% for non-MAP mycobacteria on fecal culture, 0.0 to 12.9% for seropositivity on ELISA-A, and 0.0 to 12.9% for seropositivity on ELISA-B. At the herd level, 3 of 17 herds had at least 1 animal fecal culture positive for MAP, 6 of 17 herds had at least 1 animal fecal culture positive for non-MAP mycobacteria, 13 of 17 herds had at least 1 animal positive with ELISA-A, and 9 of 17 herds had at least 1 animal positive with ELISA-B.

Table 1: Paratuberculosis ELISA and fecal culture results.
Paratuberculosis ELISA and radiometric fecal culture results for 715 Texas Longhorns from central and coastal Texas.

Fecal Culture	ELISA-A		ELISA-B	
	Negative	Positive	Negative	Positive
M. avium subsp. *paratuberculosis*	1	3	0	4
Non-MAP *Mycobacterium* spp.	68	9	69	8
Negative	610	24	622	12

ELISA-A (HerdChek®; IDEXX Laboratories)
ELISA-B (ParaChek®; Prionics)

Of the 720 animals sampled, 460 were registered allowing verification of familial relationships. Twenty-five animals (5 dams and 20 sires) appeared ≥ 20 times in the 3 generation pedigrees collected. Ancestors A, J, and Y were selected for multivariable modeling based on their univariate associations with ELISA-A paratuberculosis status

(Table 2). Confounding of the association between ancestors and offspring paratuberculosis status was not sufficiently controlled using mixed-effects models requiring the use of conditional logistic regression. The multivariable analysis estimated the odds of being positive on ELISA-A as 4.3 (95% CI, 1.3 to 14.3), 5.2 (95% CI, 1.1 to 24.7), and 4.9 (95% CI, 0.6 to 40.1) times greater if an animal had ancestors A, J, and Y, respectively, in their pedigree compared to absence of those ancestors, adjusted for presence of other ancestors and herd of residence. Ancestors P, R, and Y were selected for modeling based on their univariate associations with ELISA-B paratuberculosis status (Table 3). Significant associations were not identified for the presence of any of these ancestors in the multivariable analysis, adjusted for presence of other ancestors and herd of residence. Conditional logistic regression analysis of the association between seropositivity and the number of times that an ancestor appeared in a pedigree relative to absence of that ancestor identified ancestors A, J, O, T, and Y as significant at the $P < 0.25$ level for ELISA-A and ancestors A, P, R, and Y as significant at the $P < 0.25$ level for ELISA-B in the univariate analyses; however, only the associations for the presence of ancestor A occurring 1 time in the pedigree (OR, 5.2; 95% CI, 1.4 to 19.9) and ancestor O occurring 2 times in the pedigree (OR, 65.4; 95% CI, 3.7 to 1160.9) for results from ELISA-A were significant at the $P < 0.05$ level in the multivariable analysis. None of the ancestors demonstrated significant associations in the multivariable analysis using results from ELISA-B. In both the univariate and multivariable analyses for ELISA-A results, the point estimates for ancestors A and O demonstrated increasing odds of seropositivity as the number of

Table 2: Ancestor associations with seropositivity using ELISA-A.
Odds ratios and 95% confidence intervals for univariate conditional logistic regression models of the association between presence of an ancestor in the pedigree and paratuberculosis seropositivity for ELISA-A matched by herd of residence, and multivariable conditional logistic regression models for this association matched by herd of residence and adjusted for the presence of other ancestors. Seropositivity is defined using the S:P ratio ≥ 0.25 cut-off recommended by the manufacturer. Referent group for all comparisons is absence of the ancestor in the pedigree.

	Univariate				Multivariable			
		95% CI				95% CI		
Ancestor	OR	Lower	Upper	P value	OR	Lower	Upper	P value
A	3.05	0.97	9.61	0.06	4.25	1.26	14.33	0.02
B	1.34	0.26	6.99	0.73				
C	0.95	0.20	4.64	0.95				
D	1.32	0.36	4.82	0.67				
E	1.05	0.22	5.16	0.95				
F	1.93	0.49	7.64	0.35				
G	0.58	0.06	5.28	0.63				
H	0.51	0.06	3.99	0.52				
I	0.43	0.05	3.58	0.43				
J	3.17	0.77	13.10	0.11	5.23	1.11	24.70	0.04
K †								
L †								
M	0.52	0.08	3.43	0.50				
N	1.15	0.11	11.64	0.91				
O	2.53	0.47	13.68	0.28				
P †								
Q †								
R	1.95	0.20	18.70	0.56				
S †								
T	2.64	0.47	15.06	0.27				
U †								
V	1.12	0.13	9.43	0.92				
W †								
X †								
Y	3.61	0.50	26.24	0.21	4.87	0.59	40.10	0.14

† Analysis could not be performed because all offspring were negative on ELISA-A

Table 3: Ancestor associations with seropositivity using ELISA-B.
Odds ratios and 95% confidence intervals for univariate conditional logistic regression models of the association between presence of an ancestor in the pedigree and paratuberculosis seropositivity for ELISA-B matched by herd of residence, and multivariable conditional logistic regression models for this association matched by herd of residence and adjusted for the presence of other ancestors. Seropositivity is defined using manufacturer's recommendations. Referent group for all comparisons is absence of the ancestor in the pedigree.

	Univariate				Multivariable			
		95% CI				95% CI		
Ancestor	OR	Lower	Upper	P value	OR	Lower	Upper	P value
A	0.90	0.18	4.57	0.90				
B	1.94	0.18	21.07	0.59				
C	1.91	0.34	10.67	0.46				
D	1.44	0.24	8.62	0.69				
E	0.91	0.09	7.55	0.85				
F †								
G	1.76	0.17	18.36	0.64				
H	1.25	0.15	10.69	0.84				
I	1.22	0.15	10.14	0.86				
J	2.58	0.44	15.24	0.30				
K †								
L †								
M	0.35	0.03	3.57	0.37				
N	0.65	0.07	6.33	0.71				
O	2.94	0.32	27.31	0.34				
P	4.74	0.48	46.47	0.18	2.65	0.20	34.56	0.46
Q †								
R	6.40	0.40	102.65	0.19	3.28	0.11	94.74	0.49
S †								
T	2.29	0.22	24.17	0.49				
U †								
V	1.80	0.19	17.33	0.61				
W †								
X †								
Y	15.00	0.94	239.81	0.06	10.35	0.49	220.05	0.13

† Analysis could not be performed because all offspring were negative on ELISA-B

times the ancestor appeared in the pedigree increased. The odds of being seropositive on ELISA-A associated with ancestor A were 5.2 (95% CI, 1.4 to 19.9) and 11.6 (95% CI, 0.9 to 158.1) times greater if ancestor A appeared 1 and 2 times, respectively, in the pedigree compared to absence of that ancestor. The odds of being seropositive on ELISA-A associated with ancestor O were 1.5 (95% CI, 0.1 to 16.2) and 65.4 (95% CI, 3.7 to 1160.9) times greater if ancestor O appeared 1 and 2 times, respectively, in the pedigree compared to absence of that ancestor.

Among the 460 registered animals sampled, 106 dam-offspring pairs were identified for which ELISA-A results were available for both animals. The low number of animals within these pairs with positive ELISA-B results precluded analysis of dam-offspring associations for that test. The odds of being classified as a "suspect" or higher using the S:P $\geq$ 0.10 cut-off were 4.6 times greater (95% CI 1.0 to 20.3) if the dam was classified as a "suspect" or greater compared to dams classified as "negative" adjusted for age of offspring with herd of residence included as a random effect. Statistically significant associations were not detected using higher S:P ratio cut-offs.

Linear regression did not demonstrate evidence of confounding by herd of residence for the association between dam and offspring transformed S:P ratios adjusted for age. Therefore, herd was modeled as a random effect term to account for correlation of observations within herd. Results of the GLLAMM model demonstrated a significant positive linear association ($\beta = 0.45$; 95% CI 0.08 to 0.82) between transformed S:P ratio of the dam and offspring (Figure 1). Partitioning of the variance using mixed-effects models with the dam's transformed S:P ratio modeled as a random effect estimated that

10.7% of the variability in offspring transformed S:P ratio was due to the variability in dam transformed S:P ratio. Based on this estimate, the predicted heritability of the ELISA-A test result was 21.4%, in this population.

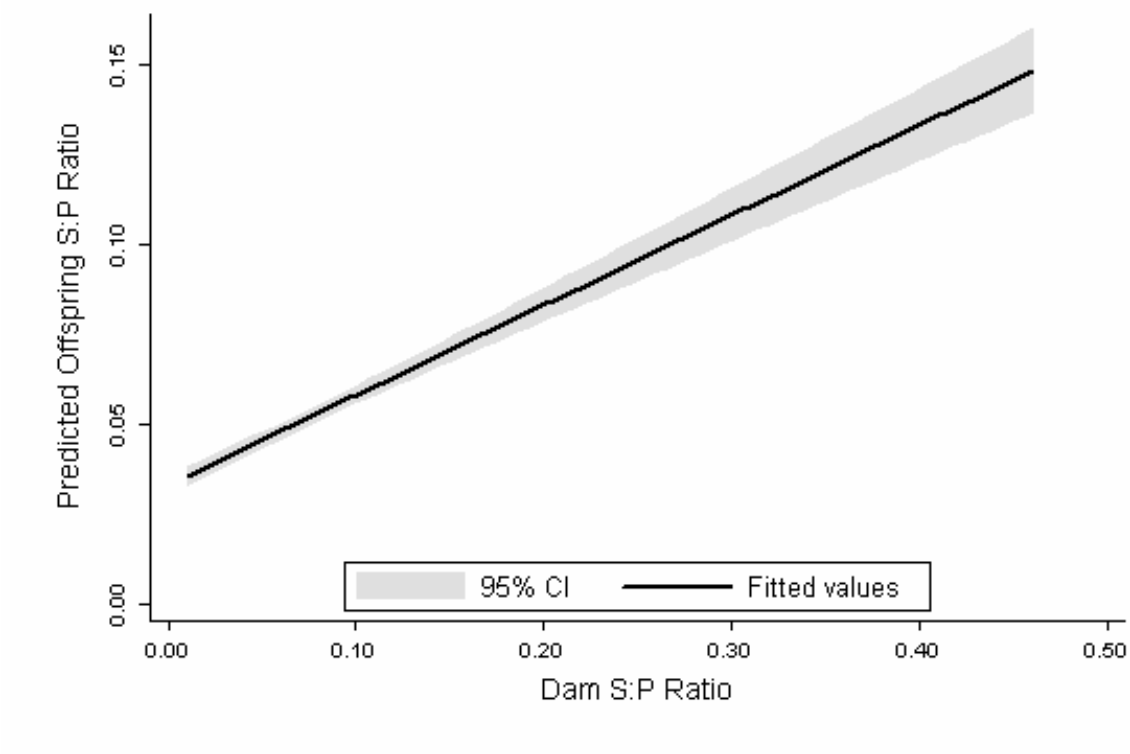

Figure 1: Linear association between dam and offspring ELISA-A S:P ratio.
Graph of predicted offspring ELISA-A S:P ratio and 95% confidence intervals for Texas Longhorn cattle given dam S:P ratio based on GLLAMM model adjusted for age of offspring and including a random effects term for herd of residence.

2.4 Discussion

There has been substantial interest in defining genetic associations with paratuberculosis status. This is due in part to the limited success achieved in paratuberculosis control programs based on diagnostic testing and culling of test positive animals. The ability to select for animals with resistance to infection or clinical disease may contribute to the success of existing control programs by reducing the population at risk and environmental contamination with MAP. Several studies have reported associations between paratuberculosis status of the dam and her offspring in dairy cattle (Koets et al., 2000; Nielsen et al., 2002b; Mortensen et al., 2004; Aly and Thurmond, 2005) and estimates of heritability of paratuberculosis in Holsteins are available (Koets et al., 2000; Mortensen et al., 2004; Gonda et al., 2006).

This study identified evidence for familial aggregation of paratuberculosis ELISA results in registered Texas Longhorn cattle. The Texas Longhorn cattle breed was selected for this study because it was anticipated that a high proportion of cattle would be registered relative to other beef breeds. Despite this unique feature, they are managed similarly and represent a model of extensively reared beef cattle for paratuberculosis research. Further, the seroprevalence in the sampled population (5%) is consistent with reports in other Texas purebred herds (Roussel et al., 2005). Increased odds of paratuberculosis seropositivity were identified with several individual ancestors in the pedigree analysis, and increased dam S:P ratio was associated with increased offspring S:P ratio. Although not significant at the manufacturer's recommended S:P ratio cut-off, the observed significant association between "suspect" or higher

classification of the dam and offspring paratuberculosis ELISA-A status suggests that offspring of animals with increased ELISA S:P ratio are at increased odds of having similarly increased S:P ratios. The inability to recognize a significant association at higher cut-offs was likely due to the small number of animals with serum ELISA S:P values > 0.25 and a lack of statistical power for testing these hypotheses.

Based on the disparity between results from microbial culture of feces for MAP and paratuberculosis ELISA results, it is likely that some of the animals with positive ELISA results were not infected with MAP. Previous reports in Texas beef cattle have identified associations between exposure of cattle to non-MAP *Mycobacterium* spp. and false-positive reactions using the ELISAs employed in this study (Osterstock et al., 2007; Roussel et al., 2007). This association limits the direct application of the observed results to control of MAP infection in beef cattle herds. However, the results of this study are important for the understanding of familial associations with paratuberculosis infection. The association between ELISA status of the dam and her offspring can be decomposed into several components: in utero infection with MAP, transmission of MAP through colostrum or milk, direct exposure of the calf to feces from an infected dam containing MAP, co-residence in a herd with exposure to MAP or non-MAP *Mycobacterium* spp, genetic control of susceptibility to MAP infection, and genetic control of humoral immune responses to *Mycobacterium* spp. The present findings are important for the identification of associations attributed to genetic control of humoral immune responses in a population with a low proportion of infected animals.

Genetic control of humoral immune responses has been extensively described in mice (Biozzi et al., 1979; Mouton et al., 1985; Puel and Mouton, 1996). Of particular importance is the fact that genetic control of humoral and cell-mediated immune responses appear to be separate and independent (Pinard-van der Laan MH, 2002). For instance, different polymorphisms in the bovine MHC are independently associated with cell-mediated and humoral responses to infection (Rupp et al., 2007). The independence of cell-mediated and humoral immune responses may be of particular importance in paratuberculosis where temporal differences are noted in onset and relative level of these components of immunity (Coussens, 2004). Initial control of MAP infection is due to a strong cell-mediated response dominated by macrophages in the intestine. Humoral responses predominate in later stages after dissemination of the infection and onset of clinical signs. To date, familial and genetic associations with MAP infection have largely been limited to classification of disease state using serum and milk ELISAs (Nielsen et al., 2002b; Mortensen et al., 2004; Gonda et al., 2006; Gonda et al., 2007b). The findings in this study in Texas beef cattle indicate that paratuberculosis antibody status should be used with caution in identifying specific genetic associations with MAP infection because antibody response is generally not associated with control of the infection. Further, it may be possible and advantageous to select for resistance to MAP infection independent of antibody status.

The analysis of the number of times an ancestor appears in an individual animal's pedigree and the observed increasing odds of seropositivity associated with inbreeding for certain ancestors supports the contention that genotypes exist that are associated with

susceptibility to seropositivity. The probability that an individual and an ancestor share a hypothetical allele associated with disease or seropositivity increases in inbred individuals. Therefore, we expect the risk of disease or seropositivity to increase if that ancestor possessed the associated allele. This would be further supported if the disease and test status of these ancestors were known. However, none of these ancestors were sampled in this study and most are foundation sires and dams that are no longer in production. It is likely that for some ancestors there was insufficient statistical power to detect an association with 95% confidence due to limited numbers of offspring in the sample population. Therefore, we cannot conclude that additional associations do not exist in this breed.

The limited sample numbers, particularly the small number of dam-offspring pairs, combined with the low seroprevalence restricted some aspects of the analysis. We failed to identify a significant association using traditional cut-offs for ELISA-A in this study and generally observed imprecise effect estimates evidenced by the wide 95% CIs. We were able to estimate the association between paratuberculosis ELISA-A status of the dam and her offspring using a reduced cut-off corresponding to the "suspect" or higher classification (Collins, 2002). This classification system was derived for use in dairy cattle and is associated with recommendations for retesting and increased likelihood of infection when compared to cattle classified as "negative" (S:P < 0.10).

The results of this research support our hypothesis that familial aggregation of paratuberculosis ELISA results exists in beef cattle. While the results of this study cannot be attributed exclusively to MAP infection, they do support inherited

susceptibility patterns for humoral immune responses to *Mycobacterium* spp. including MAP. Further, these results demonstrate that false-positive serum ELISA reactions include familial predisposition to the development of antibodies following mycobacterial exposure. In light of the evidence suggesting that humoral immune responses to *Mycobacterium* spp. is subject to familial aggregation in beef cattle, paratuberculosis control programs in herds with evidence of false-positive serological reactions associated with non-MAP *Mycobacterium* spp. should employ confirmatory tests and consider familial associations when making culling decisions. Additionally, genetic selection based on paratuberculosis ELISA status may decrease seroprevalence, but may have no effect on paratuberculosis control in herds with exposure to non-MAP *Mycobacterium* spp. Further work is necessary to describe paratuberculosis risk in beef cattle associated with test status of the dam and to identify specific genetic elements that contribute to differences in disease susceptibility.

2.5 Conclusions

The results of this study support the hypothesis that the offspring of animals with increased ELISA S:P ratios are more likely to have increased ELISA S:P ratios than the offspring of dams with lower S:P ratios. This is similar to findings in dairy cattle, but differs from previous reports due to the likelihood of false-positive serological responses due to exposure to non-MAP *Mycobacterium* spp. Further study is warranted to determine how the observed associations are influenced by the proportion of positive serological reactions attributed to non-MAP *Mycobacterium* spp. observed in this study.

3. ASSESSING FAMILIAL AGGREGATION OF PARATUBERCULOSIS IN BEEF CATTLE OF UNKNOWN PEDIGREE

3.1 Introduction

Paratuberculosis, commonly referred to as Johne's disease, is a chronic granulomatous enteritis of ruminants associated with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection. The disease is associated with significant economic losses in the U.S. cattle industries (Stabel, 1998; Ott et al., 1999; Pence et al., 2003; Lombard et al., 2005; Losinger, 2005; Losinger, 2006). Animals generally become infected during the first year of life, but do not develop clinical signs until later (Larsen et al., 1975). There is evidence to suggest that genetics play a role in disease resistance in dairy cattle populations. Heritability of paratuberculosis in dairy cattle has been estimated to be between 6 and 15% (Koets et al., 2000; Mortensen et al., 2004; Gonda et al., 2006), and associations between paratuberculosis status and polymorphism within bovine chromosome 20 have been described (Gonda et al., 2007b). However, all of these studies were performed in dairy cattle for which pedigree data were available and some of these studies (Gonda et al., 2006; Gonda et al., 2007b) utilized a limited number of sires to identify associations between genotype and disease status.

Genetic influence on paratuberculosis status in beef cattle has not been well described. Evidence of increased risk of seropositivity in *Bos indicus* cattle breeds has been identified in 2 studies (Roussel et al., 2005; Elzo et al., 2006). We have shown that familial aggregation of paratuberculosis ELISA-status exists in Texas Longhorn cattle

based on the association between ancestors and test-status of offspring and have identified a positive linear association between the antibody status of the dam and her offspring (See Section 2).

Investigations of genetic contributions to infectious disease risk often begin with describing familial aggregation. Familial aggregation is generally defined as the increase in odds of disease among family members of affected individuals compared to those of the unaffected (Liang and Beaty, 2000). Describing this association can help to estimate the proportion of disease risk associated with genetic factors and begin to differentiate environmental and genetic components. Studies of genetics and infectious disease in cattle populations have generally been performed using either candidate gene approaches or animals of known pedigree. There are important limitations to these 2 approaches. Genetic resistance to infectious disease is likely to be polygenic with a few exceptions including resistance to colibacillosis in swine (Sellwood, 1979; Rapacz and Hasler-Rapacz, 1986). This suggests that candidate gene approaches are unlikely to sufficiently describe genetic contributions to disease risk. The efficiency of candidate gene studies may be limited if the genotypes of sampled individuals do not reflect the genetic distribution of target populations. Candidate gene studies are also subject to bias due to preconceived knowledge of pathophysiology of infectious diseases. For example, recognition of the role of cell-mediated immune responses in paratuberculosis in sheep motivated preliminary studies investigating candidate gene polymorphism and paratuberculosis status (Reddacliff et al., 2005). Significant associations between polymorphism in natural resistance associated protein 1 (formerly NRAMP1; presently

referred to as SLC11a1) and major histocompatability complex (MHC) loci were noted, but identification of these associations may be biased by selection of loci sampled rather than reflecting the importance of these loci in susceptibility. Family-based studies can help identify familial aggregation and begin to discriminate between genetic and environmental components of disease risk. However, populations of cattle suitable for family-based association studies are limited to registered purebreds and animals with extensive production records including herds that use artificial insemination or embryo transfer. It is likely that these subpopulations of cattle do not reflect the larger genetic pool of the commercial cattle industries, raising concern regarding selection bias in these studies and external validity. Additionally, differences in management of purebred animals or herds with extensive pedigree records may introduce selection bias if the management of these herds also influences risk of exposure to infectious agents. An alternative approach would be to describe distributions of disease in cattle by evaluating genetic similarity among cattle that are sampled based on disease risk rather than availability of pedigree records.

The objective of this case-control study was to compare the odds of being test-positive for paratuberculosis using antibody tests and bacteriologic culture of feces between groups of genetically similar beef cattle defined using Bayesian methods for describing population substructure. Additionally, we sought to validate this approach for describing familial aggregation by comparing the cluster results with known familial structure obtained from pedigree records.

3.2 Materials and methods

3.2.1 *Animals and diagnostic tests*

Two groups of beef cattle in central Texas were identified for paratuberculosis testing and genetic analysis. Texas Longhorn breeders within a 150 mile radius of College Station, TX were surveyed from a list obtained from 1 of 2 Texas Longhorn breed directories. The survey instrument was delivered by mail and solicited information regarding herd size, history of paratuberculosis, willingness to participate in the Texas Voluntary Johne's Disease Program, and willingness to participate in this research project. Of the herds willing to enroll in the state program and the research project, all herds with greater than 15 animals 2 years of age or older and all herds with a history of clinical paratuberculosis, animals with undifferentiated chronic diarrhea and weight loss, or paratuberculosis test-positive animals were selected for sampling. A second group of herds were identified for sampling based on diagnosis of paratuberculosis within the herd through admission of individual animals to the Texas Veterinary Medical Center or by referral from practicing veterinarians in Texas. These herds were selected independent of beef cattle breed or availability of pedigree records. Pedigree data, when available, was collected for all registered animals in both groups of herds from breed registry certificates. Pedigree data was used to identify parent-offspring pairs included among all cattle sampled.

Within all herds, all animals 2 years of age or older were selected for diagnostic testing. Fecal samples were collected from the rectum with a single-use, non-lubricated

rectal sleeve. Blood samples were collected by coccygeal or jugular venipuncture. Whole blood was preserved on FTA® Classic cards (Whatman International, Newton Center, MA) for genotyping. The remaining blood sample was submitted for paratuberculosis testing using 2 commercially available ELISA test kits: ELISA-A (HerdChek®, IDEXX Laboratories Inc, Westbrook, ME) and ELISA-B (Parachek®, Prionics, Schlieren, Switzerland). Test results for ELISA-A were converted to S:P ratios by taking the difference between the sample optical density (OD) and the mean of duplicate negative control ODs and dividing by the difference between the means of the positive and negative control ODs. Individual sample results were dichotomized into positive or negative using the S:P ratio cut-off of 0.25, as recommended by the manufacturer. Results from ELISA-B were classified as positive if the difference between the assay cut-off value and the sample OD was greater than 0. The cut-off value for this assay is determined by adding 0.1 to the mean OD of the duplicate negative controls on each run of the assay. Fecal samples were submitted for radiometric fecal culture in liquid medium as previously described (Collins et al., 1990). Briefly, the medium was supplemented with mycobactin J, egg yolk suspension, and antimicrobials. Fecal samples were decontaminated with 1.0% hexadecylpyridinium chloride and concentrated via filtration. The resulting filter membrane was placed into radiometric culture medium (BACTEC 12B medium, BD Diagnostic Systems, Franklin Lakes, NJ) and evaluated weekly for growth using an ionization detector (BACTEC 460, Johnston Laboratories, Towson, MD). A PCR assay for the IS900 gene insertion element was used to identify MAP when acid-fast organisms were cultured. Mycobacterial isolates

negative for IS900 were classified as non-MAP *Mycobacterium* spp. and further characterization was not performed.

3.2.2 *Genotyping*

All parent-offspring pairs identified from breed registry certificates or production records were selected for genotyping regardless of paratuberculosis test-status for validation of the clustering method. Cases selected for genotyping were defined as all animals with at least 1 positive paratuberculosis test result; ELISA-A, ELISA-B, or fecal culture. For each case, 3 controls were matched by herd and randomly selected from those animals in the herd with ELISA-A S:P ratios ≤ 0.0. Parent-offspring pairs used to validate the clustering methods were not eligible for inclusion as cases or controls. DNA samples were obtained from whole blood stored on FTA® Classic cards collected during herd sampling.

A biopsy punch was used to harvest a 1.2 mm sample from the FTA® Classic cards for each animal selected for genotyping. Punches were placed in 200 µL wells in 96 well plates for processing. All plates included positive and negative control wells consisting of purified DNA from an animal of known genotype and PCR reagents only, respectively. Preparation of the punches was performed using a modification of previously described methods (Thacker et al., 1999). All samples were prepared for genotyping using 3 consecutive washes with 150 µL of FTA® wash (Whatman International, Newton Center, MA) with 15 minutes of incubation on a rocker panel at room temperature and 30 seconds of vortexing every 5 minutes during incubation for

each wash. Samples were then rinsed using 150 µL of Tris EDTA (1mM Tris, 0.1mM EDTA, pH 7.4) with 15 minutes of incubation on a rocker panel at room temperature and 30 seconds of vortexing every 5 minutes during incubation. Punches were dried in a forced-air hood for 2.5 to 3 hours.

A panel of microsatellites developed for parentage testing was used to genotype all parent-offspring pairs, cases, and controls (Schnabel et al., 2000). The panel consisted of 2 multiplexed PCR reactions with 6 microsatellites each (Table 4). Multiplex 1 conditions consisted of the following in a 5 µL reaction: 1.2 mm DNA punch, 0.375 units Taq polymerase (Promega GoTaq®, Promega Corp, Madison, WI), 2.5 mM deoxynucleotide triphosphate (dNTP), 17.5 mM $MgCl_2$, 0.5 µL 10X MasterAmp (Epicentre Biotechnologies; Madison, WI), 1.5 µL 5X buffer (Promega 5X Colorless GoTaq® Flexi Buffer, Promega Corp, Madison, WI), and 0.4 to 1.35 µM reverse and fluorescently labeled forward primers. Multiplex 2 conditions differed only in primer concentrations; 0.4 to 0.9 µM reverse and fluorescently labeled forward primers. All PCR reactions were carried out in a GeneAmp PCR system 9700 thermocycler (Applied Biosystems, Foster City, CA). Thermocycler events included: 3 minutes at 96° C; 4 cycles of 20 seconds at 96° C, 30 seconds at 58° C, and 1.5 minutes at 65° C; 1 minute at 96° C, 1 minute at 54° C, 20 minutes at 65° C; maintained at 4° C. One µL of PCR product from each sample was treated with 10 µL deionized formamide and denatured for 4 minutes at 96° C after addition 0.3 µL of internal size standard (MapMarker ROX, BioVentures, Murfreesboro, TN). The resulting products were genotyped on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Genotype was

determined using known alleles for cattle (Schnabel et al., 2000) and commercially available software (GeneMapper version 3.7, Applied Biosystems, Foster City, CA). Allele assignments were reviewed manually by a single investigator (JO) to confirm genotype and identify samples of insufficient quality that required repeated genotyping.

Table 4: Microsatellite loci for genotyping cattle.
Microsatellite loci, their respective fluorescent dyes, chromosomal position, and the number of alleles reported in cattle (Schnabel et al., 2000)

	Marker	Label	Position	# Known Alleles
Multiplex 1				
	BM17132	FAM	19	11
	BMS1862	VIC	24	13
	BMS410	NED	12	12
	BMS510	VIC	28	12
	BMS527	FAM	1	12
	RM372	VIC	8	8
Mulitplex 2				
	BM1225	NED	20	9
	BM1706	FAM	16	10
	BM1905	NED	23	11
	BM2113	FAM	2	8
	BM4440	NED	2	11
	BM720	VIC	13	13

3.2.3 *Data analysis*

Cluster analysis was performed on all parent-offspring pairs, cases, and controls to define population substructure using commercially available software (Pritchard et al., 2000). Clusters were defined using a Bayesian clustering algorithm based on allele

frequency. Cattle were assigned to their respective clusters probabilistically given their genotype and the allele distributions within clusters. The model for this analysis assigns a probability for the animal to have an individual allele given an assumed population of origin, the allele frequency in that population of origin, and the degree of admixture. The populations of origin and allele frequencies in the population of origin are unknown quantities and the degree of admixture can be estimated from the data after assigning the total number of clusters. The allele probabilities within each cluster were modeled assuming a Dirichlet distribution. This is a multivariate generalization of the beta distribution that allows the sum of allele probabilities for individual loci over all clusters and the sum of probabilities for all alleles at each locus within a cluster to equal 1. Markov chain Monte Carlo (MCMC) simulation techniques were used to sample from the probability of the population of origin for the allele, the allele frequency within that population, and the proportion of a given individual's genome that arose from that population given the genotypic data. The probability that an individual belonged to a given cluster could then be inferred based on the results of the MCMC sampling methods.

Clustering was performed using a burn-in of 20,000 iterations followed by a MCMC of 50,000 iterations. Analysis was performed for 1 to 22 clusters, corresponding to the number of herds, and replicated 5 times within each number of clusters (k) to assess stability of the model for the assigned cluster number. The post hoc estimate of model fit in this program (L(K)) is the average log likelihood of the data for all MCMC iterations within a given k less ½ the variance of this mean. A regression line was plotted

for replicates of models for each *k* using a fractional polynomial prediction of L(K). Optimal number of clusters was determined by considering the maximum value of L(K) for each *k* and by using the delta-*k* (Δ*k*) statistic proposed by Evanno et al. (2005). This statistic is derived by measuring the second order rate of change in L(K) between successive runs at increasing *k*. The optimal *k* can be selected corresponding to the maximum absolute value of Δ*k*. Each individual's probability of assignment to the clusters was calculated at the optimal *k* based on allele frequency of the respective clusters and the genotype of the individual.

Validity of the cluster assignment was assessed by evaluating the proportion of known parent-offspring pairs assigned to the same cluster at the optimal *k* and was compared to these proportions for other values of *k* using a 2-sided test for homogeneity of proportions. A chi-square test was performed to compare the number of parent-offspring pairs assigned to the same cluster with the number of pairs of individuals that would be assigned to the same cluster by chance assuming binomial sampling. Distribution of clusters within and among breeds and herds was also evaluated to ensure that analysis of population substructure discriminated between herds and identified genetic differences beyond the level of herd where appropriate.

The odds of having at least 1 positive paratuberculosis test result among the 2 ELISAs and fecal culture were compared among clusters using conditional logistic regression of case and control samples conditioned on herd of residence. Conditional logistic regression was performed with commercially available software (Intercooled

Stata version 9.2 for Windows, StataCorp LP, College Station, TX) and a P value < 0.05 was used to assess statistical significance.

3.3 Results

3.3.1 *Sampling and diagnostic tests*

Surveys were mailed to 762 Texas Longhorn breeders in central and coastal Texas. The total number of respondents was 147 (19.3%) from which 17 herds containing a total of 721 animals ≥ 2 years of age were selected for sampling. Of the 721 animals sampled, 715 had complete diagnostic test results including both ELISAs and fecal culture, 4 (0.6%) were fecal culture positive for MAP, 77 (10.7%) had other *Mycobacterium* spp. cultured from their feces, 36 (5.0%) were positive on ELISA-A, and 24 (3.4%) were positive on ELISA-B. Of those animals with negative fecal cultures, 24 (3.8%) were seropositive with ELISA-A and 12 (1.9%) were seropositive with ELISA-B.

Five additional beef cattle herds were sampled including 1901 animals ≥ 2 years of age. These included 3 crossbred herds of varying *Bos indicus* X *Bos taurus* proportions (n =747), 1 herd with Brahman, Shorthorn, and crossbred cattle (n =381, 44, and 523, respectively), and 1 herd with Angus cattle (n =206). All herds had reported clinical cases of paratuberculosis within the previous 2 years. Within these herds, 1,892 animals had complete diagnostic test results including 5 (0.3%) fecal culture positive for

MAP, 39 (2.1%) fecal culture positive for other *Mycobacterium* spp., 59 (3.1%) positive on ELISA-A, and 62 (3.3%) positive on ELISA-B.

3.3.2 *Genotyping and cluster analysis*

Genotypes were established for 592 animals sampled for paratuberculosis testing. This included 299 animals in known parent-offspring pairs, 105 animals positive on at least 1 test for paratuberculosis, and 288 herd-matched, test-negative controls. The complete complement of 3 controls per case could not be achieved due to genotyping difficulties for some samples of insufficient quality. Analysis of population substructure identified several potential optimal numbers of clusters based on the reflection of the regression line associated with a plot of L(K) for replicates of the analysis over *k* assignments from 1 to 22 (Figure 2) and the Δk statistic (Figure 3). Cluster assignments for *k* equal to 9 was selected for analysis based on the proximity of the L(K) regression curve to the transition to stability at that value of *k* and the similarity of Δk for *k* equal to 9 compared to 7 and 11 clusters.

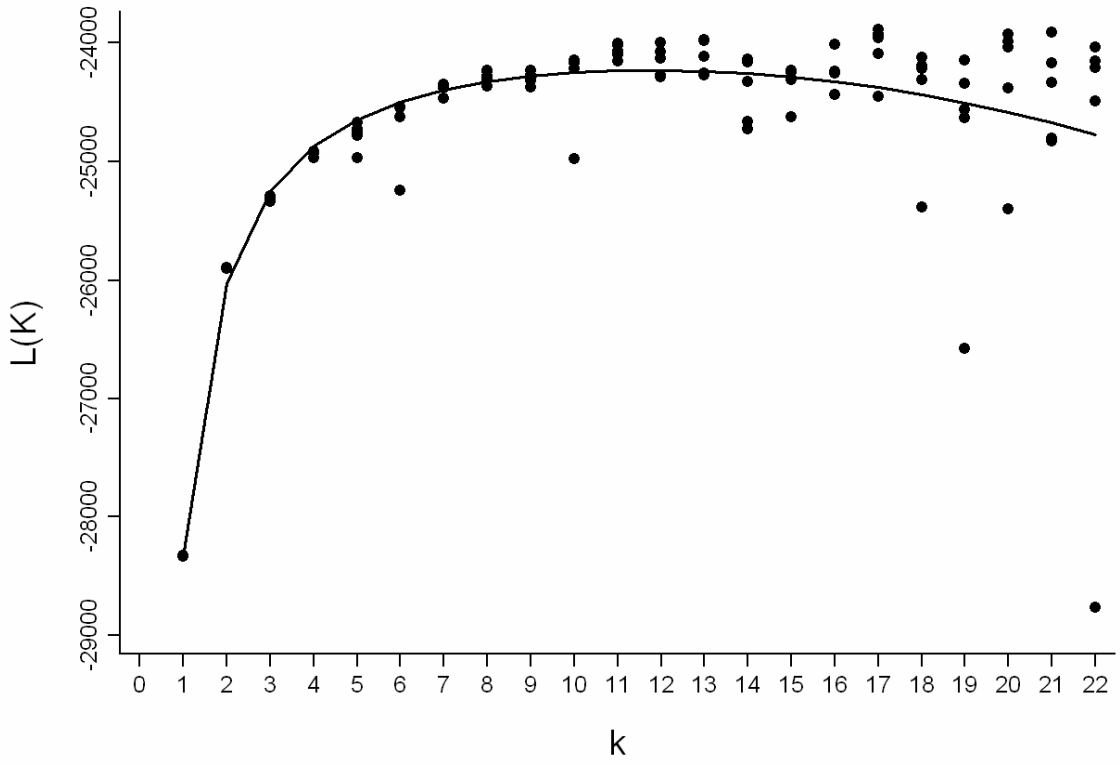

Figure 2: L(K) for different numbers of clusters.
Mean log likelihood less ½ the variance of the mean (L(K)), number of assigned clusters (*k*), and regression line for replicates of a Bayesian model to assign individual beef cattle sampled for paratuberculosis testing to clusters of genetically similar individuals based on allele frequency for 12 microsatellite loci.

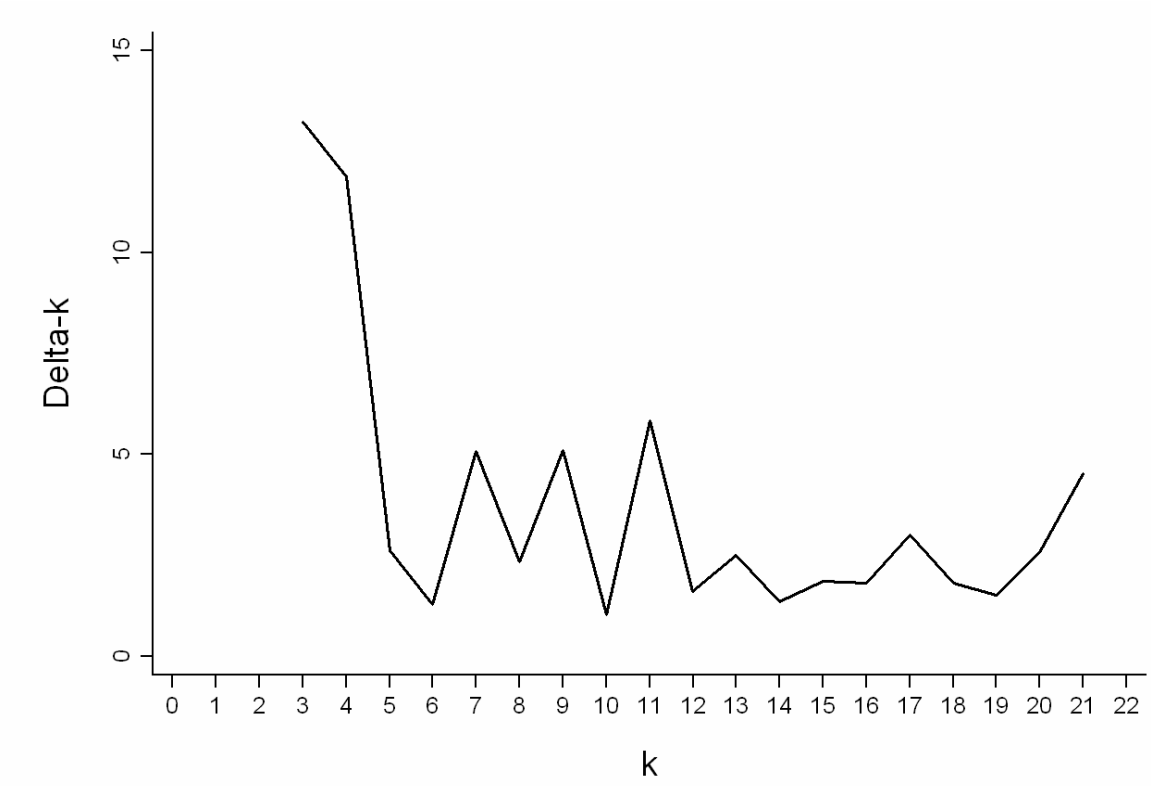

Figure 3: Delta-*k* for replicates of different number of clusters.
Evanno et al.'s delta-*k* (Δ*k*) over different numbers of assigned clusters (*k*) for identification of population substructure in beef cattle sampled for paratuberculosis testing. Larger values of Δ*k* are indicative of improved model fit for corresponding levels of *k*.

Cluster assignment appeared to appropriately differentiate breeds and define genetic diversity beyond the level of herd (Tables 5 & 6). Of the known parent offspring pairs that were genotyped, the use of 9 clusters assigned both parent and offspring to the same cluster for 46 of 55 (83.4%) of sire-offspring pairs and 110 of 149 (73.8%) of dam-offspring pairs. The number of pairs assigned to the same cluster was significantly greater than would be expected by chance (7.3 and 19.7 for sire and dam-offspring pairs,

respectively) for the number of individuals genotyped and this number of clusters ($P < 0.001$). The proportion of parent-offspring pairs assigned to the same cluster appeared consistent for values of *k* equal to 4, 7, 9, and 11. Significant differences were not detected for the proportion of sire-offspring pairs or dam-offspring pairs assigned to the same cluster between any of the values of *k* evaluated.

3.3.3 *Associations with paratuberculosis*

Proportion of paratuberculosis test-positive cattle in each cluster assignment varied among the 9 clusters (Table 7). For the conditional logistic regression models, the cluster with the lowest proportion of seropositive animals when excluding parent-offspring pairs was selected as the referent cluster. Significant increases in odds of having at least 1 positive paratuberculosis test result were identified for clusters 2 (OR 36.4; 95% CI 3.1 to 430.4), 7 (OR 7.4; 95% CI 2.2 to 25.0), and 9 (OR 5.9; 95% CI 1.8 to 19.4) compared to the cluster with the lowest proportion of positive test results (cluster 1) (Table 8). Cluster 7, containing a total of 49 animals, included 5 of the 9 animals fecal culture positive for MAP (1 Longhorn and 4 Brahman cattle). No other cluster contained more than 1 animal fecal culture positive for MAP.

Table 5: Distribution of cattle breeds within clusters.
Distribution of breeds within clusters of genetically similar beef cattle defined on the basis of allele frequency for 12 microsatellites.

	Cluster									
Breed	1	2	3	4	5	6	7	8	9	Total
Angus	4	0	0	2	3	0	0	0	0	9
Brahman	0	0	0	0	0	0	13	0	100	113
Longhorn	14	72	20	9	3	120	2	67	0	307
Shorthorn	0	0	2	0	10	0	0	0	0	12
Crossbred	25	0	10	38	30	1	37	2	8	151
Total	43	72	32	49	46	121	52	69	108	592

Table 6: Distribution of cluster assignments within herds.
Distribution of cluster assignments defined on the basis of allele frequency for 12 microsatellites within herds of residence. Breeds (LH = Texas Longhorn; BRAH = Brahman; SH = Shorthorn; XB = *Bos indicus* X *Bos taurus* cross; ANG = Angus)

		Cluster									
Herd	Breeds	1	2	3	4	5	6	7	8	9	Total
1	LH	0	9	1	1	0	19	0	9	0	39
2	LH	0	1	0	0	0	4	0	4	0	9
3	LH	0	0	0	0	0	7	0	6	0	13
4	LH	0	1	0	0	0	6	0	13	0	20
5	LH	0	2	0	0	0	2	0	0	0	4
6	LH	0	3	1	0	0	13	0	7	0	24
7	LH	5	0	9	1	1	9	1	5	0	31
8	LH	8	0	4	1	1	6	1	1	0	22
9	LH	1	13	3	4	0	2	0	0	0	23
10	LH	0	3	1	0	0	4	0	0	0	8
11	LH	0	1	0	0	0	9	0	2	0	12
12	BRAH, SH, XB	10	0	5	24	34	1	45	2	105	226
13	LH	0	0	0	0	0	15	0	2	0	17
14	LH	0	34	0	0	0	3	0	3	0	40
15	LH	0	0	0	2	0	4	0	3	0	9
16	LH	0	4	0	0	1	10	0	6	0	21
17	XB	2	0	0	1	0	0	0	0	0	3
18	XB	2	0	3	7	3	0	3	0	2	20
19	ANG	4	0	0	2	3	0	0	0	0	9
20	XB	11	0	4	6	3	0	2	0	1	27
21	LH	0	1	1	0	0	1	0	5	0	8
22	LH	0	0	0	0	0	6	0	1	0	7
Total		43	72	32	49	46	121	52	69	108	592

Table 7: Paratuberculosis test results by cluster.
Numbers of paratuberculosis positive and negative beef cattle assigned to each cluster and proportion of positive cattle in each cluster for known parent-offspring pairs, cases, and controls. Cattle were defined as paratuberculosis positive if samples were positive for MAP antibody on either commercial ELISA or if MAP was isolated from feces.

	Cluster									
Paratuberculosis Status	1	2	3	4	5	6	7	8	9	Total
Parent-Offspring Pairs										
Negative	0	59	4	5	1	77	1	42	0	189
Positive	0	4	1	0	0	3	0	2	0	10
	0.00	0.06	0.20	0.00	0.00	0.04	0.00	0.05	0.00	0.05
Cases and controls										
Negative	37	3	19	36	38	28	32	21	74	288
Positive	6	6	8	8	7	13	19	4	34	105
	0.14	0.67	0.30	0.18	0.16	0.32	0.37	0.16	0.31	0.27

Table 8: Association between paratuberculosis test status and cluster assignment. Results of conditional logistic regression model comparing odds of being positive for MAP antibody on either commercial ELISA or microbial culture of feces for MAP matched by herd. The referent group is the cluster with the lowest proportion of positive test results among the 9 clusters excluding parent-offspring pairs.

		95% CI		
Cluster	OR	Lower	Upper	P Value
2	36.4	3.1	430.4	0.004
3	3.4	1.0	12.0	0.056
4	2.0	0.6	6.8	0.241
5	1.9	0.5	6.7	0.334
6	3.7	0.9	14.5	0.060
7	7.4	2.2	25.0	0.001
8	1.6	0.3	8.5	0.587
9	5.9	1.8	19.4	0.003

3.4 Discussion

Identifying familial aggregation of disease is an important step in identifying genetic differences in susceptibility to infectious disease. Familial aggregation also helps to differentiate genetic and environmental influences on disease susceptibility and to identify populations with disparate disease risks for future study. Studies aimed at describing familial aggregation in cattle populations have traditionally relied upon animals of known pedigree. For some infectious diseases, the study of animals with pedigree information may introduce selection bias as other management factors associated with disease prevalence may also be associated with herds of pedigreed animals. Examples would include selection for dairy cattle from farms with detailed

production records or for purebred beef cattle populations. These operations may also be more likely to purchase cattle from other operations, a risk factor for introduction of paratuberculosis into the herd (USDA, 2002). Alternatively, these herds may have more familiarity with paratuberculosis and may be more likely to have control programs in place.

This study identified increased odds of positive paratuberculosis results in some clusters of beef cattle defined on the basis of genetic similarity rather than pedigree data. Although insufficient numbers of animals with MAP positive fecal culture results were available for statistical analysis, 5 of 9 animals with positive fecal cultures were in the same cluster supporting the aggregation of paratuberculosis within this group of animals. The proportion of known parent-offspring pairs assigned to the same cluster indicated that performing cluster analysis using microsatellite data yields potentially similar results as would have been obtained from pedigree information while avoiding potential selection biases. This method appears to be able to differentiate genetic similarity beyond the herd level and may be particularly useful in describing familial aggregation in cattle that are extensively traded among producers, as is observed in Texas Longhorn cattle, where genetic similarity may spread across herds.

The clustering approach employed here based on allele frequencies has been applied to studies of genetic differentiation in humans and chickens (Rosenberg et al., 2001; Rosenberg et al., 2002). Results of these studies indicate that these methods more accurately reflect known population structure compared to genetic distance-based methods (Rosenberg et al., 2001). To our knowledge, this clustering framework has not

been used to establish clusters of genetically similar individuals with the intent of comparing proportions of diseased animals between clusters. A potential pitfall to this approach is increase in the overall experiment-wise error by applying multiple statistical models in deriving the effect estimate. Additionally, this could introduce misclassification bias if the results of the clustering models yielded inaccurate assignment of individuals to cluster groups. Misclassification bias could also be introduced if the results of the genotyping included incorrectly assigned alleles. The allele frequency-based cluster models used here, however, are not without limitation. Selection of the optimum value for *k* is somewhat subjective and model fit can not be readily tested statistically between models for different levels of *k*. We used 2 criteria for selecting the optimal *k* and both methods yielded similar results. It is likely that some datasets evaluated using these methods would have multiple values for *k* that similarly fit the data because of cluster subdivision with increasing values of *k*. Another limitation of these models is lack of convergence for individual runs at a given *k*. MCMC sampling methods may not converge and may not sufficiently explore all potential modes causing inferences based on these models, specifically the proportion of an individual's genome that would be assigned to a given cluster, to be unstable. We attempted to reduce the likelihood of relying on spurious model results by replicating each model run 5 times for each value of *k*.

The clusters with significantly increased odds of positive paratuberculosis test results demonstrate some additional associations and features of this approach to describing familial aggregation. Cluster 2, the cluster with the highest relative odds, was

comprised exclusively of Texas Longhorn cattle. The cattle included in this cluster represent 10 different herds demonstrating the utility of this approach in identifying genetic structure across herds. This supports the dissemination of genetic factors associated with paratuberculosis test-status as environmental factors would be expected to play a smaller role in the odds of having a positive paratuberculosis test result with so many environmental exposures represented. Clusters 7 and 9 were comprised predominantly of Brahman and Brahman-cross cattle. Cluster 7 contained cattle from 5 herds including 2 Texas Longhorns, 13 Brahmans, and 37 crossbred animals with substantial proportions of *Bos indicus* ancestry. Similarly, cluster 9 contained cattle from 3 herds including 100 Brahman cattle from a single herd and 8 animals from 3 herds with substantial *Bos indicus* ancestry. The increased odds of having at least 1 positive paratuberculosis test result observed in these groups supports the findings in 2 other studies that have identified increased prevalence of paratuberculosis seropositivity in *Bos indicus* breeds of cattle (Roussel et al., 2005; Elzo et al., 2006).

The results of this study should be interpreted with caution regarding impacts on paratuberculosis control programs. The majority of the animals classified as paratuberculosis test-positive had positive results for 1 or both serum ELISAs, but did not have MAP isolated from their feces using radiometric fecal culture methods. Additionally, 116 animals had non-MAP *Mycobacterium* spp. isolated from their feces. Previous reports have found that environmental and experimental exposure to non-MAP *Mycobacterium* spp. is associated with false-positive serum ELISA results in Texas beef cattle (Osterstock et al., 2007; Roussel et al., 2007). Therefore, the increased odds

observed in some of the clusters in this study could reflect genetic differences associated with humoral responses to *Mycobacterium* spp. rather than infection with MAP. The influence of the findings of these 2 studies on the perceived increase in prevalence of paratuberculosis seropositivity in Brahman cattle is unclear, but may be associated with the geographical distributions in Texas of these cattle breeds and the *Mycobacterium* spp. associated with false-positive antibody responses.

Matching animals selected for genotyping by herd and removing a subset of animals (parent-offspring pairs) from the analysis introduces some limitations to this study. Animals with ELISA-A S:P ratios ≤ 0.0 were preferentially selected as controls to increase the likelihood of genotyping animals with both highly resistant and susceptible genotypes for positive paratuberculosis test results. The use of conditional logistic regression to control for the matching variable, in this case herd, creates data that becomes sparse once stratified. This causes imprecise estimates as evidenced by the wide confidence intervals for many of the effect estimates. However, animals within a herd may have similar genetic composition and environmental exposures prompting attempts to control for this potential confounder in the analysis. The effect of the selection bias imposed by this matching criterion would likely be bias towards the null, assuming individuals within a herd are more likely to be genetically similar. Larger sample sizes may help to eliminate the problem of sparse data after stratifying by herd; however, paratuberculosis prevalence in beef cattle is generally low (Thorne and Hardin, 1997; Dargatz et al., 2001b; Hill et al., 2003; Pence et al., 2003; Roussel et al., 2005) and a consistent impediment to studies of the disease in these cattle. Removing parent-

offspring pairs from the analysis contributed to this problem by reducing sample size since some cattle among these pairs were paratuberculosis test-positive. They were removed in this study to allow unbiased assessment of the validity of the method for describing familial aggregation, but this removal would likely be unnecessary in future studies using similar methods.

The results of this study offer a novel approach to the assessment of familial aggregation of infectious disease in cattle populations that may be less subject to selection bias than study designs that rely on the identification of animals of known ancestry. We also have identified significant differences in the odds of having positive paratuberculosis test results among the clusters supporting a genetic basis for these differences in beef cattle.

3.5 Conclusions

Clustering of beef cattle based on microsatellite allele frequency data was used to define groups of genetically similar individuals and demonstrated that these methods can be used reliably in the absence of pedigree information to describe familial aggregation of infectious disease. Three clusters were identified that had significantly greater odds of having at least 1 positive paratuberculosis test result. One of these clusters (OR 36.4; 95% CI 3.1 to 430.4) was comprised exclusively of Texas Longhorn cattle. Two of these clusters (OR 7.4; 95% CI 2.2 to 25.0 and OR 5.9; 95% CI 1.8 to 19.4) contained large proportions of Brahman and *Bos indicus* crossbred cattle. The results of this study demonstrate that familial aggregation of paratuberculosis test-status exists in beef cattle

and support the hypothesis that *Bos indicus* cattle breeds may be predisposed to MAP infection or seropositivity.

4. PREDICTING PARATUBERCULOSIS ELISA STATUS IN BEEF CATTLE BASED ON FAMILIAL AND HERD-LEVEL RISK FACTORS

4.1 Introduction

Paratuberculosis is a granulomatous enteritis of ruminants associated with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection. The disease is distributed worldwide and has been associated with substantial economic losses in the U.S. cattle industry (Stabel, 1998; Ott et al., 1999; Pence et al., 2003; Lombard et al., 2005; Losinger, 2005; Losinger, 2006). Paratuberculosis control programs in beef cattle herds in the U.S. have focused primarily on culling of clinically affected animals, implementation of management changes aimed at reducing environmental contamination and calf exposure to MAP, and screening using routine diagnostic testing to identify subclinically infected animals (USDA, 2002). The latter approach has many limitations including inaccuracy of commercially available diagnostic tests (Collins et al., 1991; Sockett et al., 1992; Reichel et al., 1999; Whitlock et al., 2000; Nielsen et al., 2001; Dargatz et al., 2001a; Kalis et al., 2002; Collins et al., 2005; McKenna et al., 2005a; McKenna et al., 2005b) and prolonged latency of infection with a large proportion of subclinically infected animals that cannot be detected. One of the most common diagnostic methods used in herd surveillance programs is detection of serum antibody using commercial ELISAs. These tests are favored due to the rapid availability of results when compared to fecal culture, low cost, and relative ease of sample collection. However, commercially available ELISAs have been reported to have low sensitivity

(Dargatz et al., 2001a; Collins et al., 2005; McKenna et al., 2005a), due in part to a delay in onset of circulating antibody in infected animals. In some herds, exposure to non-MAP *Mycobacterium* spp. in the environment has been associated with decreased specificity of commercial tests for MAP antibody (Osterstock et al., 2007; Roussel et al., 2007).

Recent reports have identified genetic associations with paratuberculosis antibody status in dairy cattle (Nielsen et al., 2002b; Mortensen et al., 2004; Gonda et al., 2006). Heritability estimates of paratuberculosis status range from 6% to 10.2% (Koets et al., 2000; Nielsen et al., 2002b; Mortensen et al., 2004; Gonda et al., 2006) and evidence for a quantitative trait locus (QTL) on *Bos taurus* chromosome 20 (BTA20) has been reported (Gonda et al., 2007b). In beef cattle, a positive linear association between the MAP antibody status of the dam and the status of her offspring was identified (See Section 2). Additionally, familial aggregation of paratuberculosis in beef cattle has been demonstrated using microsatellite markers and cluster analysis supporting genetic contributions to risk of seropositivity (See Section 3). In light of anecdotal evidence supporting increased risk of infection in offspring of infected animals, reported risks associated with in utero infection (Seitz et al., 1989; Sweeney et al., 1992b), and reported genetic associations with paratuberculosis test status, a commonly utilized approach to paratuberculosis control is to cull the offspring of clinically affected or seropositive animals, regardless of the test status of the offspring. While this practice may reduce herd seropositivity, the effectiveness of this control measure may be greatly reduced if a substantial proportion of serological reactions are false-positives associated

with non-MAP *Mycobacterium* spp. or other factors unrelated to MAP infection. Further, inappropriate culling of offspring based upon test status of the dam may increase economic losses attributed to paratuberculosis due to loss of genetically valuable animals.

The objective of this cross-sectional study was to evaluate the association between paratuberculosis ELISA status of the dam and ELISA status of her offspring in beef cattle adjusted for individual and herd-level factors including within herd prevalence of MAP and non-MAP *Mycobacterium* spp.

4.2 Materials and methods

4.2.1 *Animals and diagnostic testing*

Beef cattle were selected for sampling as previously described (unpublished data). In brief, beef cattle herds in central and coastal Texas were identified for sampling through breeder survey, practicing veterinarian referrals, and admissions to the Texas Veterinary Medical Center. All herds selected for sampling agreed to participate in the Texas Voluntary Johne's Disease Program and provided written consent for enrollment in the research project approved by the Clinical Research Review Committee in the Texas A&M University College of Veterinary Medicine and Biomedical Sciences.

All animals ≥ 2 years of age within selected herds were tested for paratuberculosis. Blood samples were collected from the coccygeal or jugular vein into plain evacuated tubes (BD Vacutainer®, BD Biosciences, Franklin Lakes, NJ). Fecal

samples were collected from the rectum and were stored in individual plastic bags (Whirl-Pak®, Advantec MFS Inc, Dublin, CA). Samples were shipped to a commercial laboratory (Johne's Testing Center, Madison, WI) within 24 hours of sample collection. Serum was harvested from collected blood samples and analyzed for MAP antibody using a commercially available ELISA kit (HerdChek®, IDEXX Laboratories Inc, Westbrook, ME). ELISA results were converted to S:P ratios by taking the difference between the sample optical density (OD) and the mean of duplicate negative control ODs and dividing by the difference between the means of the positive and negative control ODs. Individual sample results were dichotomized into positive or negative using the S:P ratio cut-off of 0.25, as recommended by the manufacturer. Fecal samples were processed for radiometric culture as previously described (Collins et al., 1990). Briefly, the medium was supplemented with mycobactin J, egg yolk suspension, and antimicrobials. Fecal samples were decontaminated with 1.0% hexadecylpyridinium chloride and concentrated via filtration. The resulting filter membrane was placed into radiometric culture medium (BACTEC 12B medium, BD Diagnostic Systems, Franklin Lakes, NJ) and evaluated weekly for growth using an ionization detector (BACTEC 460, Johnston Laboratories, Towson, MD). A PCR assay for the IS900 gene insertion element was used to identify MAP when acid-fast organisms were cultured. Mycobacterial isolates negative for IS900 were classified as non-MAP *Mycobacterium* spp. and further characterization was not performed.

4.2.2 *Data analysis*

Pedigree and production records were utilized to identify dam-offspring and sire-offspring pairs for which both animals in the respective pairs had available diagnostic test results and to verify age. Animals from herds without sufficient production records to verify age or parent ELISA status were assigned missing values for these variables. Values for missing data were imputed within the modeling procedure. ELISA status of the sire and dam were imputed using random assignment from a Bernoulli distribution with a success probability of 0.03 based on the individual animal-level prevalence of 3% that was reported in a similar beef cattle population using the same ELISA kit (Roussel et al., 2005). Missing values for age were randomly assigned using the distribution of observed ages in the sample population defined using commercially available software for fitting distributions to data (@Risk, Palisade Corp, Ithaca, NY). Herd-level seroprevalence, fecal prevalence of MAP, and fecal prevalence of non-MAP *Mycobacterium* spp. were calculated from collected specimens and available diagnostic test data.

Individual paratuberculosis antibody status was modeled using Bayesian mixed-effects logistic regression and Markov chain Monte Carlo (MCMC) techniques with available software (WinBUGS version 1.4, Medical Research Council, Cambridge, UK) (Appendix B). For the purposes of this study, the primary exposure of interest was the ELISA status of the dam. Additional individual-level covariates selected for modeling included age of animal and ELISA status of the sire. Herd-level covariates selected for modeling included herd size, herd history of animals with clinical paratuberculosis,

ELISA seroprevalence, MAP fecal prevalence, and non-MAP *Mycobacterium* spp. fecal prevalence. Herd of residence was included as a random effect to account for correlation of observations at the herd level. The random effect term for herd was modeled assuming a normal distribution with a common variance following a gamma distribution. Random assignment of imputed values for missing data was performed independently within each iteration of the MCMC procedure.

Informative priors for regression coefficients were derived using univariate conditional logistic regression for individual-level covariates and ordinary logistic regression for herd-level covariates (Intercooled Stata version 9.2 for Windows, StataCorp LP, College Station, TX). Coefficients were assumed to be normally distributed with mean equal to the point estimate from the logistic regression analysis and standard deviation equal to the reported standard error of the point estimate (Table 9). Convergence of the model was assessed by monitoring the number of iterations required for the Gelman-Rubin statistic of 2 simultaneous chains with different starting points for unknown parameters to converge to a value of 1. Additionally, the densities of the informative priors were compared to the posterior densities to evaluate changes in the means and width of the 95% intervals. The number of iterations necessary for convergence was set as the burn-in and an additional 10,000 iterations were used to derive effect estimates.

Table 9: Priors for regression coefficients.
Priors for regression coefficients derived using conditional logistic regression for individual-level covariates and logistic regression for herd-level covariates. All priors were modeled using a normal distribution.

Variable	Mean	Standard Deviation
Age	-0.015	0.054
Dam ELISA status	0.234	1.147
Sire ELISA status	-36.280	3.900
Herd non-MAP *Mycobacterium* spp. status	0.000	10.000
Herd MAP prevalence	25.772	11.553
Herd seroprevalence	18.358	2.500
Herd history of paratuberculosis	-0.2770	0.217
Herd size	0.0002	0.200

The full model with all individual and herd-level covariates was fit and the mean regression coefficients were recorded. Sensitivity analysis was performed to assess the influence of the priors on the posterior densities of unknown parameters by replacing the prior density in the model statement with a non-informative (flat) prior distribution (μ = 0; S.D. = 100). Parameters that demonstrated a substantial change in posterior mean including change in direction of effect or change in the posterior mean greater than 25% were reevaluated. Variables with missing values that demonstrated substantial sensitivity to the prior densities due to lack of power in the sample were removed from the model. Herd-level parameters with substantial sensitivity were evaluated for violations of linearity assumptions and, if present, were modeled as categorical variables using indicator variables for different categories or as binary variables if only 2 levels were

considered. Variables were then removed from the model individually and the resulting mean effects were compared to the full model to assess for confounding of the association between remaining predictor variables and offspring ELISA status. Confounding was defined as a change in the exponentiated parameter estimate greater than 15% after removal of the term from the model. Covariates that confounded any association between predictors and offspring ELISA status were retained in the final model. Sensitivity analysis was repeated at each step of the model building procedure.

Model selection was further performed by removing terms from the model and comparing the deviance information criteria (DIC) between nested models (Spiegelhalter et al., 2002; Celeux et al., 2006). The model with the smallest DIC was selected for parsimony where a decrease in DIC of greater than 5 was deemed a significant improvement in model fit. The DIC for each model was derived from the deviance reported by WinBUGS for a given model (Model 1) using informative priors for regression coefficients and the deviance for the same model fit replacing the regression coefficients of fixed and random effects (Model 2) with constants corresponding to the posterior means for the unknown parameters from the previous model. DIC was calculated as the deviance of Model 2 subtracted from twice the deviance reported for Model 1 (Celeux et al., 2006). The difference in model fit was compared between full and nested models by subtracting the DIC of the reduced model from the full model. ELISA status of the dam and variables that confounded any association between a given predictor and offspring ELISA status were forced into the model and not subject to removal based on change in DIC. Convergence was reevaluated and sensitivity analysis

was repeated following determination of the final model. Means of the posterior densities and 95% credibility intervals derived from the 2.5th and 97.5th percentiles of the posterior distribution were exponentiated to report odds ratios.

4.3 Results

Samples were collected from 2,621 animals ≥ 2 years of age from 22 beef cattle herds in central and coastal Texas. Mean herd size was 119 animals and ranged from 14 to 948 animals. Definitive age was available on 569 animals with a mean of 6.8 years (S.D. 3.9, range 2 to 18 years). Within the sample population, 2,540 had fecal culture results including 11 (0.4%) animals culture positive for MAP and 116 (4.6%) animals culture positive for non-MAP *Mycobacterium* spp. ELISA results were available for 2,616 including 96 (3.7%) that were seropositive. Mean within-herd prevalence of MAP based on fecal culture was 0.5% (range 0 to 4.1) and mean within-herd fecal prevalence of non-MAP *Mycobacterium* spp. was 8.0% (range 0 to 71.2). Mean within-herd seroprevalence was 4.7% (range 0 to 15.4). Pedigree records identified 157 and 56 dam-offspring and sire-offspring pairs, respectively, for which ELISA status was known for each member of the pair. One of 8 dam-offspring pairs with ELISA positive offspring had an ELISA positive dam and 11 of 149 pairs with ELISA negative offspring had ELISA positive dams. None of the 3 sire-offspring pairs with ELISA positive offspring had ELISA positive sires and 2 of 53 pairs with ELISA negative offspring had ELISA positive sires. The proportion of seropositive dams and sires was 7.6% and 3.6%, respectively.

Convergence was achieved following 20,000 iterations and parameter estimates were based on an additional 10,000 iterations after burn-in. Sire status was removed from the model following initial sensitivity analysis. The posterior density was highly dependent upon the prior density and the effect was strongly protective ($\beta = -36$). Investigation revealed that this variable was perfectly correlated with dam ELISA status introducing multicollinearity into the model. Further, the relatively small number of observations was dominated by the prior density. Herd fecal non-MAP *Mycobacterium* spp. prevalence when modeled as a continuous variable was also sensitive to the prior and the direction of effect changed between informative and non-informative prior density models. Evaluation of quartiles of this variable demonstrated that the effect of this variable was not linear in the log odds. Herd fecal non-MAP *Mycobacterium* spp. prevalence was subsequently recoded as a categorical variable with 4 levels (<5%; ≥5% and <10%; ≥10% and <20%; and ≥ 20%). This term also demonstrated sensitivity to the prior and the direction of effect changed between informative and non-informative prior density models. In these models, the random intercepts for the 2 herds with the highest proportion of animals shedding non-MAP *Mycobacterium* spp. changed sign between informative and non-informative priors. The influence of these random effects on the effect of non-MAP *Mycobacterium* spp. prevalence and its sensitivity to prior densities indicated that this categorization was not adequate. Fecal non-MAP *Mycobacterium* spp. prevalence was then modeled with only 2 levels (<5%; ≥5%). The 5% cut-off corresponds to the proportion of animals with non-MAP *Mycobacterium* spp. isolations from feces in herds without unusual proportions of ELISA positive animals (Roussel et

al., 2007). This binary classification did not demonstrate over-sensitivity to prior densities and was retained in the model.

Significant decrease in DIC was noted following removal of herd history of clinical paratuberculosis from the model. Confounding of the association between other predictors and offspring ELISA status was not detected following removal of herd history. Removal of other terms from the model did not result in significant improvement in model fit. The final model included age, dam ELISA status, herd size, herd ELISA prevalence, herd fecal MAP prevalence, herd fecal non-MAP *Mycobacterium* spp. prevalence modeled as a categorical variable with 2 levels, and a random effect term for herd. Odds ratios and 95% credibility intervals were derived from the parameter estimates for individual and herd-level covariates (Table 10). Results of sensitivity analysis using non-informative priors on all parameters did not show substantial changes in means of the posterior densities (Table 11).

Associations with high posterior probabilities were observed for herd seroprevalence (OR 1.21 per 1% increase; 95% CI 1.18 to 1.24) and herd fecal MAP prevalence (OR 1.28 per 1% increase; 95% CI 1.20 to 1.37). Associations between age, herd size, and herd fecal non-MAP *Mycobacterium* spp. status were small and not highly probable at the $P < 0.05$ level. The odds of having a positive ELISA result were estimated to be 1.35 times greater for the offspring of dams with positive ELISA results, but this association was not highly probable ($P = 0.688$).

Table 10: Coefficient estimates with informative priors.
Odds ratios (OR) and 95% credibility intervals from a mixed-effects Bayesian logistic regression model for predicting paratuberculosis ELISA status using individual and herd-level covariates in Texas beef cattle.

Variable	Mean β	OR	95% Credibility Interval		P value
			Lower	Upper	2-sided[†]
Age (incr. 1 year)	-0.04	0.96	0.87	1.06	0.488
Dam ELISA status	0.30	1.35	0.27	5.95	0.688
Herd non-MAP *Mycobacterium* spp. status	-0.41	0.66	0.34	1.30	0.226
Herd MAP prevalence (incr. 1%)	24.76	1.28	1.20	1.37	<0.0001
Herd seroprevalence (incr. 1%)	19.01	1.21	1.18	1.24	<0.0001
Herd size (incr. 10 head)	0.0001	1.00	0.99	1.01	0.683

[†] 2-sided P values were derived for parameter estimates by estimating the proportion of the posterior density > 0 and < 0 for parameters with negative and positive means, respectively, and multiplying by 2

Table 11: Coefficient estimates with non-informative priors.
Odds ratios (OR) and 95% credibility intervals from a mixed-effects Bayesian logistic regression model with non-informative prior densities for predicting paratuberculosis ELISA status using individual and herd-level covariates in Texas beef cattle.

			95% Credibility Interval	
Variable	Mean β	OR	Lower	Upper
Age (incr. 1 year)	-0.05	0.95	0.85	1.06
Dam ELISA status	0.23	1.25	0.05	11.45
Herd non-MAP *Mycobacterium* spp. status	-0.51	0.60	0.27	1.35
Herd MAP prevalence (incr. 1%)	20.36	1.23	1.05	1.43
Herd Seroprevalence (incr. 1%)	22.42	1.25	1.17	1.35
Herd size (incr. 10 head)	0.0002	1.00	0.99	1.01

4.4 Discussion

Predicting the paratuberculosis status of offspring in beef cattle would be beneficial in herds with existing paratuberculosis control and management programs. One of the impediments to the success of paratuberculosis control programs is the long period of time following infection prior to the onset of clinical signs or the ability to detect infection using currently available diagnostic tests. Previous attempts to model the transmission of paratuberculosis in cattle have been in dairy herds (Collins and Morgan, 1991; Groenendaal et al., 2002; Groenendaal and Galligan, 2003) and are largely aimed at predicting economic impact of various control strategies. One report simulated the transmission of paratuberculosis in seasonally calving beef cattle herds following introduction of an infected animal (Humphry et al., 2006). This model emphasized the role of the environment in transmission of paratuberculosis including persistence of MAP in the environment and bacterial density in a contaminated environment. One of the weaknesses of this model was that it does not explicitly include the influence of paratuberculosis status of the dam on risk of infection in the calf. Terms were included to account for vertical transmission of MAP in utero in clinical and non-clinical cows, but this is likely to play a relatively minor role in transmission in beef cattle herds due to the low prevalence of infection and the small proportion of congenitally infected calves (Seitz et al., 1989; Sweeney et al., 1992b). Additional maternal factors that may influence the risk of infection in calves include transmission of MAP through colostrum or milk (Sweeney et al., 1992a; Streeter et al., 1995), genetic factors associated with

resistance to infection, and increased opportunity for the dam to expose her calf to higher levels of feces contaminated with MAP when the dam is actively shedding the organism.

A previous study in beef cattle identified positive associations between paratuberculosis ELISA status of the dam and her offspring (unpublished data). In that study, the odds of being classified as a "suspect" or greater (Collins, 2002) using a commercially available paratuberculosis ELISA were 5 times greater if the dam was also classified as "suspect" or greater compared to offspring of dams classified as "negative". However, the influence of herd-level environmental risk factors on this association was not evaluated other than to include herd of residence as a random effect to account for correlation of observations within herd. The present study identified increased odds of seropositivity with increasing herd seroprevalence and herd fecal MAP prevalence. A probable association was not detected between the paratuberculosis antibody status of the dam and her offspring and the mean effect estimate was smaller (OR 1.35) than observed previously (unpublished data). The smaller point estimate in this study may be due to the higher ELISA S:P ratio cut-off employed here (0.25) compared to the previous study (0.10). The smaller effect of dam ELISA status in a model that included herd-level covariates may indicate that the observed association in the previous study reflected similarities in the shared environment of the dam and her calf rather than direct dam-to-calf risk factors including genetic susceptibility to seropositivity, congenital infection, or infection via colostrum and milk.

A factor that limits the interpretation of this model with regard to paratuberculosis infection is the exposure to non-MAP *Mycobacterium* spp. in the

environment that have been associated with false-positive serological reactions using paratuberculosis ELISAs (Osterstock et al., 2007; Roussel et al., 2007). It is likely, given the disparity between the number of animals with MAP isolated from feces and the number of animals with positive ELISA results, that some of the ELISA positive cattle were not infected with MAP. We controlled for herd-level exposure to these *Mycobacterium* spp. by including fecal prevalence in the model. However, fecal prevalence of non-MAP *Mycobacterium* spp. could not be modeled as a linear term and the inclusion of this variable as a binary exposure may not have adequately modeled its effect. In the present model, herds with fecal non-MAP *Mycobacterium* spp. prevalence ≥ 5% had a decreased odds of being ELISA positive, though not statistically probable. This appears counterintuitive given the reported association between environmental mycobacteria and paratuberculosis ELISA results (Osterstock et al., 2007; Roussel et al., 2007). There are likely 2 factors that contributed to this result. Seroprevalence and herd-level non-MAP *Mycobacterium* spp. exposure status are correlated. Therefore, inclusion of both terms in the model would not be necessary. However, given previous observations in paratuberculosis testing in Texas beef cattle, we felt it was necessary to retain both terms in the model assuming the term for non-MAP *Mycobacterium* spp. was eligible for inclusion given other model building criteria. The negative association between herd non-MAP *Mycobacterium* spp. status and offspring paratuberculosis ELISA status may also be due to differences in specific *Mycobacterium* spp. present within herds. Previous reports have demonstrated that the proportion of specific *Mycobacterium* spp. isolated varies among herds and different *Mycobacterium* spp. are

associated with varying effects on paratuberculosis ELISA status (Osterstock et al., 2007; Roussel et al., 2007). Speciation of non-MAP *Mycobacterium* spp. isolates was not performed in this study, but if the isolates between herds varied with respect to the likelihood of causing false-positive ELISA results, this may have affected the association as measured in this model.

In the present model, age did not have a statistically probable effect and the point estimate was less than 1 (OR 0.96 per 1 year increase; 95% CI 0.87 to 1.06). This may appear counterintuitive given our understanding of the progression of paratuberculosis and the age at onset of seropositivity observed in dairy cattle. There are 2 likely explanations for the observed negative and non-probable effect of age in the present study. The distribution of known ages in the sample population included more animals greater than 10 years of age than has been typically observed in dairy cattle where the association between age and infection status or seropositivity has been reported (Kalis et al., 1999; Nielsen et al., 2002a; van Schaik et al., 2003; Nielsen and Ersboll, 2006; Nielsen and Toft, 2006). The positive ELISA results associated with non-MAP *Mycobacterium* spp. may also have affected the effect of age. An age dependent susceptibility to seropositivity associated with environmental mycobacteria has not been reported.

Theoretically, a more complete evaluation of the influence of genetic factors on the odds of paratuberculosis seropositivity would have been achieved if ELISA status of the sire could have been included in the model. In the present study, a small number of offspring had serological data available for the sire. The modeling methods employed

here randomly assigned serological status using the estimated prevalence in the beef cattle population to missing sire observations and, subsequently, the priors on the regression coefficient for sire status imposed substantial influence on the measured effect. There was also evidence of multicollinearity between sire and dam ELISA status. Further study using beef cattle populations with more complete sire information may be useful to further define this relationship. The limited number of observations with serological data for the dam may have similarly influenced the effect of dam in the present model and provided insufficient power to detect a difference. However, similar influence of the prior on the regression coefficient for dam ELISA status was not observed.

The use of a Bayesian framework for evaluating exposures associated with paratuberculosis ELISA status has several strengths and limitations. Bayesian methods allow incorporation of prior information into the model using the observed data to update our preconceived knowledge concerning estimates of effect. The Bayesian framework allows all observations to contribute to the estimation of the associations between predictor variables and the outcome, in spite of missing data. Typical maximum likelihood methods would limit the estimation of effect to those observations with complete data. In this model, we used prior information regarding seroprevalence to impute missing serological data for the sire and dam and the empirical sample distribution of age to impute missing values for age. The iterative process employed in MCMC methods allows this imputation to occur randomly for each iteration within the limits of the specified distributions. However, application of inappropriate prior

knowledge may bias the observed associations. For paratuberculosis in beef cattle, there is limited information regarding the associations between ELISA status and familial and environmental risk factors. Had substantive information been available, it would have been preferable to use that information in deriving priors for the parameters in the model, as is typically done, rather than estimates of effect from analysis of the data using maximum likelihood methods. Further, deriving priors from data used to construct the model may unduly influence precision of the parameter estimates and introduce bias due to underestimated variability. An additional limitation of the methods employed here is the difficulty in selecting the appropriate terms to include in the model. Model selection in mixed-effects models, particularly with missing data, is very sensitive to the measure of model fit applied, the level of hierarchy to which it is applied, and is computationally intensive (Celeux et al., 2006). For the present model, we used a derivation of DIC and interpreted model fit at the individual animal level. Alternative measures of model fit may have yielded different results.

Based on these results, beef cattle producers and veterinarians should not emphasize the serological status of the dam when making paratuberculosis control decisions within herds, particularly regarding the culling of untested animals. This is in contrast to previous findings in beef (unpublished data) and dairy cattle (Koets et al., 2000; Nielsen et al., 2002b; Mortensen et al., 2004) where the influence of the paratuberculosis status of the dam was deemed significant in predicting the paratuberculosis status of offspring. The difference between the relative importance of the association between the dam and her offspring observed here in beef cattle and

previous reports in dairy cattle is unknown. Intuitively, the longer typical duration of exposure between a cow and her calf in beef cattle operations would be expected to increase the effect of the dam on offspring ELISA status compared to dairy cattle operations where the calf has limited exposure to the dam. However, calves in beef cattle operations also have prolonged contact with the entire adult population and the adult herd environment during the period when they are most susceptible to infection (Larsen et al., 1975). Dairy calves are typically removed from the maternity pen shortly after birth and are managed in an environment physically separated from the adult herd. Therefore, management of dairy calves is typically less variable between calves on the same farm and among calves on different farms with the exception of the paratuberculosis status of the dam and exposure to MAP from the dam that may occur during the immediate post-partum period including contamination of the udder or immediate environment with feces containing MAP. Further study is needed to determine the specific differences between beef and dairy calf management that contribute to the differences in risk of seropositivity associated with ELISA status of the dam.

4.5 Conclusions

Higher odds of seropositivity were observed for increases in herd seroprevalence and herd fecal MAP prevalence. A probable association between the ELISA status of the dam and her offspring was not observed. The results of this study indicate that the paratuberculosis ELISA status of beef cattle may not be influenced by the ELISA status

of the dam when controlling for herd-level exposures. Therefore, ELISA status of the dam should not be used when making culling decisions in paratuberculosis control programs in beef cattle herds when herd-level MAP and ELISA prevalence is available.

5. CONCLUSIONS

5.1 Conclusions from present work

There is increasing evidence to support genetic contribution to susceptibility of cattle to paratuberculosis (Koets et al., 2000; Nielsen et al., 2002b; Mortensen et al., 2004; Gonda et al., 2006; Gonda, 2006; Gonda et al., 2007b). To date, these investigations have been limited to dairy cattle, specifically the Holstein breed. The results of the work presented in this dissertation serve as an initial investigation of familial predisposition to paratuberculosis seropositivity and MAP infection in beef cattle on the basis of serum antibody using commercially available paratuberculosis ELISAs. In Section 2, a positive association was observed between the ELISA S:P ratio of the dam and her offspring. Additionally, the offspring of dams with increased S:P ratio were more likely to have similarly increased S:P ratios using dichotomies derived from a classification scheme proposed for this ELISA test (Collins, 2002). The results presented in Section 3 demonstrate the presence of familial aggregation of paratuberculosis test status in beef cattle of unknown pedigree. This novel approach to identify differences in prevalence of paratuberculosis test-positive cattle within "families" utilized microsatellite data to define groups of genetically similar individuals that was modeled as proxy for familial information. Finally, a model was constructed to predict paratuberculosis ELISA status in beef cattle using information from animal and herd-level variables including familial paratuberculosis status and within-herd prevalence of seropositivity. This model can be utilized to derive predicted ELISA status

of offspring of seropositive cattle to aid in culling decisions implemented as part of paratuberculosis control programs. However, the ELISA status of the dam was not associated with a statistically significant increase in odds of positive ELISA in her offspring in this model and the mean effect estimate was smaller (OR 1.35; 95% CI 0.27 to 5.95) than found in Section 2.

There are 2 substantial challenges to assessing familial aggregation of paratuberculosis in beef cattle using serological data that were encountered in these studies. First, the prevalence of paratuberculosis in beef cattle is generally low (Braun et al., 1990; Thorne and Hardin, 1997; Dargatz et al., 2001b; Hill et al., 2003; Pence et al., 2003; Roussel et al., 2005) and a prevalence of approximately 3% was identified in the sample population used in these projects. The sampling strategy used for all of the studies presented here attempted to increase the seroprevalence in the sample population by preferentially selecting herds with histories of clinical cases, but did not appear to effective at identifying a sample population with a prevalence higher than what has been reported for Texas purebred cattle herds (Roussel, et al., 2005). The low prevalence of paratuberculosis in beef cattle creates a burden in study design due to the sample size required to identify significant differences in disease prevalence or risk associated with familial or herd-level risk factors. In these studies, insufficient power likely limited the ability to find some associations between paratuberculosis ELISA status of the dam and her offspring and the paratuberculosis status of offspring and the presence of a given ancestor in the pedigree as significant. This also affects the precision of effect estimates

as evidenced by the generally wide 95% confidence intervals. Therefore, the results of this work should be repeated in larger populations to confirm the findings reported here.

A second challenge encountered in the studies detailed here is the high proportion of seropositive animals that were MAP fecal culture negative. Previous work has demonstrated that exposure to non-MAP *Mycobacterium* spp. present in the environment may cause false-positive reactions when using the ELISAs employed in these studies (Osterstock et al., 2007; Roussel et al., 2007). The association between these *Mycobacterium* spp. and paratuberculosis ELISA results has to date only been reported in beef cattle and there likely are geographical associations that influence the frequency with which this exposure is encountered in cattle herds. Studies of the geographic distribution of similar *Mycobacterium* spp. in the eastern U.S. suggest that the prevalence of these bacteria increases in areas surrounding water and more southern areas appear to have higher prevalence of these bacteria in soil compared to northern areas (Falkinham et al., 1980). The implication of this exposure in Texas beef cattle herds is that the results of the work presented here must be interpreted narrowly as association with paratuberculosis ELISA status rather than MAP infection status. This is an important distinction and limits the contribution of this work to our understanding of familial associations with paratuberculosis susceptibility. However, this work does contribute to our understanding of the contribution of familial associations to humoral immune responses to *Mycobacterium* spp. Presumably, the reported association between paratuberculosis status of the dam and her offspring in dairy cattle includes genetic control of susceptibility to infection with MAP, development of serum antibodies to

MAP antigen associated with progression of infection, development of pathological lesions and associated clinical manifestations of paratuberculosis, and transmission from the dam through ingestion of colostrum or milk containing MAP (Sweeney et al., 1992a; Streeter et al., 1995) and in utero infection (Seitz et al., 1989; Sweeney et al., 1992b). While the work presented here reflects components of the perceived genetic contributions to disease risk, further study is necessary to determine the influence of non-MAP *Mycobacterium* spp. on the contribution of these results to our understanding of genetic susceptibility to paratuberculosis in beef and dairy cattle.

Genetic control of paratuberculosis susceptibility will likely become a more important area of research as our understanding of the pathogenesis of the disease improves and the use of robust platforms (ex. SNP arrays) for genome-wide association studies increases. One of the important considerations in the design and implementation of these studies will be sample selection. The ability to screen potential sample populations to identify populations of genetically similar individuals with disparate disease risk may improve the efficiency of these studies. The use of genetic markers to define familial aggregation of paratuberculosis among groups of genetically similar cattle may facilitate sample selection and help to identify groups of cattle that are likely to have the most substantial differences in genotype associated with disease risk. The methods described in Section 3 demonstrate the utility of this approach and validate the method by comparing to known familial structure. Increased odds of paratuberculosis seropositivity and preliminary evidence of increased prevalence of animals with positive fecal culture for MAP were identified for some clusters. Further detail in describing

groups of genetically similar individuals could be obtained by increasing the number of markers utilized in defining population genetic substructure. The use of these methods may be particularly important for studies of paratuberculosis susceptibility due to potential bias introduced by preferential selection of animals with known pedigree data. Pedigrees for cattle are generally only available in registered purebred herds and herds with detailed production records. Opportunity for bias may exist if herd management, particularly regarding paratuberculosis control, differs between herds with available pedigree data compared to those without such data. For instance, registered purebred herds may routinely purchase replacement animals to improve the composition of herd genetics. Purchase of replacements is an important risk factor for the introduction of infected animals into herds (USDA, 2002). Alternatively, these herds may be more familiar with paratuberculosis and may have implemented paratuberculosis control programs that include strategic biosecurity and management procedures that mitigate the risk of MAP introduction and reduce transmission within the herd in an effort to protect the offspring of genetically valuable animals.

The impact of the results of this work on design and implementation of paratuberculosis control programs may be limited, particularly in light of the proportion of seropositive animals attributed to non-MAP *Mycobacterium* spp. in the sample population. However, there are several findings that can improve the design of paratuberculosis control programs in beef cattle operations in Texas and similarly extensively managed systems. The association between the paratuberculosis ELISA status of the dam and her offspring observed in Section 2 helps to estimate the risk

associated with retaining the offspring of seropositive animals. The prediction model developed in Section 4 may also be useful to producers and veterinarians making culling decisions based in part on paratuberculosis test data. This model is designed to contribute to the prediction of paratuberculosis ELISA status in offspring of beef cattle and could be implemented prior to test age. In contrast to the association between dam and offspring paratuberculosis ELISA status observed in Section 2, a significant association was not observed between the paratuberculosis status of the dam and her offspring in a model including herd-level seroprevalence and fecal prevalence of MAP and non-MAP *Mycobacterium* spp. and the point estimate for this effect was smaller than the estimate from Section 2. Within a given beef cattle herd, the odds of having paratuberculosis ELISA positive offspring are not significantly increased for seropositive dams when adjusted for herd-level exposures.

These studies of paratuberculosis in beef cattle examined the associations between ELISA status of cattle and their ancestors, offspring, and genetically similar individuals using combinations of conditional logistic regression, mixed-effects models, and Bayesian approaches. Two factors influenced the decision to use these data analysis methods. Cattle were sampled as herds for all 3 studies with all animals ≥ 2 years of age within each herd tested for paratuberculosis. Herd of residence has 2 separate, but equally important effects on measured associations. Unmeasured herd-level factors may confound the association between paratuberculosis status of the dam and her offspring. Confounding may result in biased estimates of effect. For the analyses performed in these studies, confounding was conservatively defined as changes in the effect measure

exceeding 15% following removal of a term from the model. One of the ways to control for confounding is to stratify the analysis on levels of the confounding factor. In Sections 2 and 3, conditional logistic regression was used to control confounding by herd of residence. This approach does limit the analysis, particularly for some aspects of Section 2 where data become sparse once stratified over numerous levels of herd, reducing the power to identify statistically significant associations. Herd may also affect the results of these studies as a clustering variable due to the existing hierarchy of observations within herds. This may lead to overdispersion and inappropriate estimates of the standard errors for the measured associations. Mixed-effect models help to control for this overdispersion by incorporating a portion of the total variance, that attributed to multiple observations within the same herd, into the random effect term. This allows reporting of unbiased standard errors and allows proper interpretation of statistical tests of significance. However, if the intraclass correlation is low, as was observed in Section 2, mixed-effects models may not adequately control confounding. Therefore, despite the utility of mixed-effects models in epidemiology in herds or similar hierarchies, care should be taken to evaluate the control of confounding when hierarchical variables may also bias effect estimates using these models. In most instances, preference should be given to the reporting of unbiased effect estimates over corrected standard errors. Another potential disadvantage of mixed-effects models is that the random intercept for hierarchical terms can substantially influence the observed measures of effect, particularly if some of the higher levels of the hierarchy lie on extremes of the observed distribution of values for the fixed effects. This was observed in Section 4 where the

models appeared to computationally prefer to modify the random intercept for herds with extreme values of fecal non-MAP *Mycobacterium* spp. prevalence rather than the fixed effect for this variable. This was undoubtedly associated with the nonlinear association between ELISA status of offspring and herd-level environmental mycobacteria exposure. However, this model illustrated the need to monitor both fixed and random effect terms in model building to avoid spurious conclusions regarding effect estimates.

The Bayesian framework utilized for data analysis in Sections 3 and 4 is generally a robust approach to data analysis and helps to overcome some of the difficulties encountered with analysis of data sets with missing values for some observations. These models also allow the incorporation of prior knowledge into the analysis, using the current observations to update that knowledge. Bayesian approaches were very effective in resolving the genetic population substructure in the genotyped population in Section 3. The use of allele frequency data to establish these clusters appears to be more accurate than genetic distance-based clustering methods compared to known population structure (Rosenberg et al., 2001). However, Bayesian methods are sensitive to the prior information incorporated into model statements for unknown parameters. Inaccurate prior densities may substantially bias derived posterior effect estimates. Sensitivity analysis using models that incorporate non-informative priors into the model help to identify potential bias derived from inappropriate prior densities. For the model constructed in Section 4, very little information is available to construct prior densities for the parameters of interest for beef cattle. Prior densities were estimated

from the observed associations using maximum likelihood methods for the partial dataset. For this reason, sensitivity analysis was performed more extensively than usual for this model at numerous steps in the model building procedure.

5.2 Proposed future study

Future research in genetic susceptibility to paratuberculosis will likely focus on identifying specific genetic elements that control host response to infection or can be employed as markers for selection of resistant genotypes. These studies will likely focus on important dairy breeds due to the higher prevalence of infection within the dairy industry. However, beef cattle breeds have an opportunity to contribute to this area of research as well. Beef cattle generally remain in production longer than dairy cattle, a feature that may be particularly useful in studying a disease with a prolonged preclinical phase of infection. Exposure of beef cattle to non-MAP *Mycobacterium* spp. associated with false-positive serological reactions using commercially available ELISAs, despite posing a substantial limitation in the present work, may be important in defining different components of susceptibility to paratuberculosis. A primary goal of studies investigating genotypes associated with paratuberculosis should be to describe polymorphisms associated with susceptibility to infection, with perhaps less emphasis on immunological response of cattle that do become infected. Mounting a humoral response, while not efficient at controlling the infection (Coussens, 2004), provides the most cost effective means of identifying infected animals given currently available diagnostic testing methods. Therefore, we would not expect genetic selection against

seropositivity, regardless of the potential for exposure to non-pathogenic *Mycobacterium* spp, to be effective at eradicating paratuberculosis in any production system. Genetic selection for animals resistant to infection, but easily identified using cost effective diagnostic tests if they do become infected, would be preferable. Cattle that mount substantial humoral responses to non-MAP *Mycobacterium* spp. may help to differentiate those genes associated with humoral immune response and those associated with infection. This also underscores the need to carefully define case status and whether this classification includes the presence of pathological lesions and confirmation with fecal culture.

Additional studies must be performed within infected beef cattle herds to describe the association of the paratuberculosis status of the dam and her calf. These studies should ideally be performed in herds without history of false-positive serological reactions using commercially available diagnostic tests with high diagnostic specificity. This will likely require large sample populations to provide sufficient power for the detection of potentially small effects. Comparison of the results from these studies to reports in dairy cattle will better describe the effect of the intimate relationship between a beef cow and her calf and identify environmental and genetic components to paratuberculosis susceptibility.

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APPENDIX A

Dear (Longhorn Breeder),

You may have had experiences with Johne's disease in your cattle, or know others who have. This bacterial infection of the gastrointestinal tract can cause significant losses in your herd due to decreased production, increased culls, loss of genetic progress, and cost of veterinary care and testing. Many aspects of this disease remain unknown in beef cattle, including prevalence of infection, options for therapy, and specific risk factors for infection.

Veterinarians at Texas A&M University are working hard to address these unknowns and to help producers develop risk management and assessment tools for the control and prevention of Johne's disease. I am currently developing a research project for the study of Johne's disease in Longhorn cattle in Texas along with Drs. Allen Roussel and Geoffrey Fosgate. Our goal is to determine the risks of infection if the sire, dam, or other family members are infected. This would help to explain transmission of the disease in beef cattle, the impact of having infected animals in a herd, and support the potential for genetic components of disease resistance. Longhorns represent an ideal population for this type of study because most are of known pedigree, many come from a relatively short list of Longhorn families, and Longhorn breeders tend to keep excellent records. Longhorns have also traditionally been among the breeds we recognize as being inherently disease resistant.

Through the cooperative efforts of researchers at the Texas A&M University College of Veterinary Medicine and some of your fellow Longhorn breeders, you have been selected for a preliminary survey about Johne's disease in Longhorns in the state of Texas. This study is not affiliated with the TLBAA. The purpose of this study is to identify herds for future testing. This testing will be designed to conform to the Texas Voluntary Johne's Disease Program certification process. Testing information gathered through these herd investigations will allow us to examine the risk of infection with Johne's disease associated with infected herdmates and related cattle.

Enclosed is a brief survey about your herd and Johne's disease. As we know how busy you are, we have designed the survey to take just a few minutes. After you complete and return this survey, we will identify herds for potential testing and may contact you by telephone to further discuss opportunities for

participation in this study. All results of this survey, future conversations, and herd testing results will be kept confidential and you participation is strictly voluntary. You will not receive any direct compensation for your participation, but if your herd is enrolled, you will have the opportunity to test your herd, receive management advice, and achieve herd level certification at no cost. Alternatively, your local veterinarian may be able to provide these services on a fee basis. There is no risk to you or your herd associated with completing and returning this survey.

Please keep a copy of this information letter for your records. This research study has been reviewed by the Institutional Review Board—Human Subjects in Research, Texas A&M University. For research-related problems or questions regarding subjects' rights, you can contact the Institutional Review Board through Dr. Michael W. Buckley, Director of Research Compliance, Office of Vice President for Research at (979) 845-8585 mwbuckley@tamu.edu. By filling out and returning this survey you consent to participate in the study.

Thank you for you time and consideration in your participation in this study. With your help, we have an opportunity to address many of the challenges of Johne's disease. Please feel free to contact me if you have any questions or concerns regarding this study or your participation in it. You may also contact Dr. Allen Roussel at 979-845-3541 or Dr. Geoffrey Fosgate at 979-845-3203 for study related questions. Thanks again for your participation.

Sincerely,

Jason Osterstock, DVM
Texas A&M University
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979-845-3541
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Evaluation of individual risk for infection with *Mycobacterium avium ss paratuberculosis* associated with familial infection in Texas Longhorn cattle

A Texas Longhorn Johne's Disease Survey

Veterinarians at the Texas A&M University Veterinary Medical Center are conducting research on Johne's disease in cattle. This is a survey for Longhorn breeders about their herds. Completion of this survey is voluntary and would be greatly appreciated. After completion and return of the survey, researchers may contact you to ask other questions and to see if you would like to enroll your herd. Selection of herds for additional contact and enrollment will be randomized pending survey results. Thank you.

Name_____

Address_____

Address_____

City _____

State_____ **County** _____ **Zip**_____

Phone_____ **Alt. Phone** _____

Email _____

Do you currently raise longhorn cattle? Yes ☐ No ☐

Do you have other breeds of cattle? Yes ☐ No ☐

If so, what breeds? _____

How many longhorns >2 years of age do you have? _____

How frequently do you…

…buy cattle? Annually ☐ Every few years ☐ Never ☐

…raise your own replacements?

Annually ☐ Every few years ☐ Never ☐

Are your animals registered? Yes ☐ No ☐

Do you routinely work your cattle (ie. for vaccination, pregnancy testing, deworming, etc.)? Yes ☐ No ☐

Do you have working facilities (chute, alley, etc.)? Yes ☐ No ☐

Have you had or culled any animals with chronic diarrhea in the past 5 years?

Yes ☐ No ☐

Have you heard of Johne's disease? Yes ☐ No ☐

Have you ever tested an individual animal or your herd for Johne's disease?

Yes ☐ No ☐

Would you be interested in participating in a state supported Johne's testing and herd certification program? Yes ☐ No ☐

Would you be interested in participating in research projects investigating Johne's disease in longhorn cattle? Yes ☐ No ☐

Would you be willing to allow researchers from Texas A&M to contact you via phone or email? Yes ☐ No ☐

Thank you for your time and participation.

Jason Osterstock, DVM
Texas A&M University

APPENDIX B

```
model{

for (i in 1:2616){

indiv.status[i] ~ dbern(p[i])

logit(p[i]) <- int  + beta.dam.status*dam.status[i] + random[herd[i]] +
beta.herd.size*herd.size[i] +   beta.herd.mptbprev*herd.mptbprev[i] +
beta.herd.seroprev*herd.seroprev[i] + beta.herd.catmyco5*herd.catmyco5[i] +
beta.age*age[i]


age[i] ~ dnorm(6,5) I(2,18)
dam.status[i] ~ dbern(0.03)

}

for (j in 1:22){ random[j] ~ dnorm(0, random.tau)}
random.tau ~ dgamma(0.001, 0.001)
int ~ dnorm(0,0.001)

beta.age ~ dnorm(-.0105337,18.64844)
beta.dam.status ~ dnorm(0.234,0.872059)
beta.herd.size ~ dnorm(0.000216,5)
beta.herd.seroprev ~ dnorm(18.35783,0.4)
beta.herd.mptbprev ~ dnorm(25.77244,0.086556)
beta.herd.catmyco5 ~ dnorm(0,0.1)

beta.age.p <- step(beta.age)
beta.dam.status.p <- step( beta.dam.status)
beta.herd.size.p <- step( beta.herd.size)
beta.herd.seroprev.p <- step( beta.herd.seroprev)
beta.herd.mptbprev.p <- step( beta.herd.mptbprev)
beta.herd.catmyco5.p <- step( beta.herd.catmyco5)

}
```

VITA

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Ph.D., Biomedical Sciences, Texas A&M University, 2007

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Veterinary Intern, Colorado State University, Ft. Collins, CO, 2001
Associate Veterinarian, Red Willow Animal Clinic, McCook, NE 2002-2003
Lecturer, Texas A&M University, College Station, TX, 2004-2007

Peer Reviewed Publications:

Washburn, K., Norman, T., Osterstock, J., Respondek, T., 2007. Hairy vetch (*Vicia villosa*) toxicosis in a purebred Angus herd. Bov. Pract. 41, 60-64.

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